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Phylogeny And Systematics Of The Superfamily Diplostomoidea Poirier, 1886 (Platyhelminthes: Trematoda)

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PHYLOGENY AND SYSTEMATICS OF THE SUPERFAMILY DIPLOSTOMOIDEA POIRIER, 1886 (PLATYHELMINTHES: TREMATODA)

by

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ABSTRACT

The Diplostomoidea is a large superfamily of digeneans which possess a unique holdfast organ. Members of the superfamily are distributed worldwide and are known to parasitize a wide variety of animals, both invertebrates and vertebrates. In some cases, diplostomoideans have been associated with diseases such as ocular diplostomiasis and 'black spot' disease in fishes. The taxonomic and systematic history of diplostomoideans is complex and includes numerous revisions based on morphology and host associations. Prior to this study, the Diplostomoidea included 6 families and 16 subfamilies. Recent molecular phylogenetic analyses have revealed the Diplostomidae and the Strigeidae to be non-monophyletic and demonstrated a need for re-evaluation of the group. In the present study, diplostomoideans were collected from a diversity of intermediate and definitive hosts from around the world which resulted in the most comprehensive sample set to date. Digenean specimens were studied using morphological and molecular tools (primarily molecular phylogenies of the 28S rRNA and cytochrome c oxidase mtDNA genes) to study the interrelationships of diplostomoidean taxa, host-parasite relationships and diversity of taxa. Our results clearly demonstrate the non-monophyly of the Cyathocotylidae, Diplostomidae and Strigeidae and support the monophyletic status of the Proterodiplostomidae. Based on re-evaluation of morphological characters and results of phylogenetic analysis of partial 28S sequence, the Brauninidae is considered a junior

synonym of the Cyathocotylidae. Further, molecular phylogenies were used to re-evaluate the system of the Proterodiplostomidae. Among other findings, the current subfamily system of the Proterodiplostomidae was rejected. The results of morphological and molecular study clearly demonstrates that the diversity of diplostomoidean taxa has been underestimated, including species likely associated with ‘black spot’ disease in fish. In total, we described 1 new subfamily, 3 new genera and 5 new species of diplostomoideans with descriptions of many additional new taxa pending. Molecular phylogenetic analyses demonstrated numerous host-switching events during the evolutionary history of the Diplostomoidea along with evidence of multiple dispersal events between biogeographic realms.

CHAPTER I

INTRODUCTION

The superfamily Diplostomoidea Poirier, 1886 is a relatively diverse group of digeneans. Members of the superfamily can be distinguished from other digenean groups based on the presence of a unique holdfast organ (also referred to as the tribocytic organ). The holdfast organ may be sucker-like or bilobed depending on the group of diplostomoideans. It has been proposed that this structure plays a role in both attachment and digestion. Diplostomoideans are known to be distributed worldwide (e.g., Dubois, 1936a; Niewiadomska, 2002a–g; Blasco-Costa & Locke, 2017).

The life cycles of diplostomoideans vary among genera, however, all known life cycles require at least 2 intermediate hosts. As with other digeneans, the first intermediate host is a type of mollusk. The second intermediate hosts utilized by diplostomoidean taxa include mollusks, annelids, fishes and amphibians; in a few cases (e.g., *Strigea* Abildgaard, 1790) additional intermediate hosts may be used such as reptiles and mammals. The definitive hosts of diplostomoideans include most major lineages of tetrapods, such as fishes, snakes, crocodilians, birds and mammals (Dubois, 1936a, b; Niewiadomska, 2002a–g; Blasco-Costa & Locke, 2017).

Several members of the Diplostomoidea are known to be associated with disease in their intermediate hosts. For instance, *Crassiphiala* Van Haitsma, 1925, *Diplostomum* von Nordmann, 1832, *Ornithodiplostomum* Dubois, 1936, *Posthodiplostomum* Dubois,

1936, *Tylodelphys* Diesing, 1850 and *Uvulifer* Yamaguti, 1934 are known to be causative agents of a variety of parasitic diseases in fishes such as ocular diplostomiasis and 'black spot' disease (e.g., Hunter, 1933; Lemly & Esch, 1984; Chappell et al., 1994; Overstreet & Curran, 2004; Bullard et al., 2008; Matisz et al., 2010; McAllister et al., 2013). While less common, some diplostomoideans have been known to cause disease in their definitive hosts; for instance, *Cyathocotyle bushiensis* Khan, 1962 has been associated with massive die-offs of aquatic birds in the Midwestern United States (Gibson et al., 1972; Hoeve & Scott, 1988; Herrmann & Sorensen, 2009) and heavy infections of *Ichthyocotylurus* spp. can be lethal to their avian hosts (Swennen et al., 1979). In some cases, members of this group can cause disease in humans; for example, *Alaria* spp. are known to cause alariosis in humans, which may be lethal, as a result of ingestion of metacercariae in frogs (Tăbăran et al. 2013).

Systematic and Taxonomic History

The taxonomic history of superfamily Diplostomoidea is complex; originally members of this superfamily were placed into the family Holostomidae Blanchard, 1847 (Blanchard, 1847). Poirier (1886) later erected the Diplostomidae Poirier, 1886, while Mühling (1898) established the Cyathocotylidae Mühling, 1898. Subsequently, Railliet (1919) elevated the Holostomidae to the level of superfamily and re-named it the Strigeoidea Railliet, 1919. Poche (1926) created supersuperfamily Strigeida Poche, 1926 which included the Strigeidae Railliet, 1919 and the Cyathocotylidae. Gibson (1996) established the name Diplostomoidea for the superfamily based upon the name of the oldest family within the group (Diplostomidae). Several authors (e.g., Dubois, 1938, 1953, 1968, 1970a, b, 1982, 1987, 1989; La Rue, 1926a, b, 1957; Sudarikov, 1959, 1960a, b, 1961, 1997; Yamaguti, 1958, 1971) have introduced and disputed changes to

taxonomy, composition and systematics of diplostomoideans based on morphology and host associations. On the other hand, Shoop (1989) recognized the non-monophyly of the Diplostomidae and proposed substantial revision to the diplostomoidean system based on adult and larval morphology.

In Shoop's (1989) opinion, diplostomoideans were split across 5 sister groups. He considered the Proterodiplostomidae Dubois, 1936 and Strigeidae to be monophyletic, but viewed the Diplostomidae to consist of 3 clades; he proposed erection of 2 new diplostomid families: Neodiplostomidae Shoop, 1989 (which contained subfamilies Crassiphialinae *sensu* Shoop, 1989 and the Neodiplostominae Shoop, 1989) and the Bolbophoridae Shoop, 1989. However, Niewiadomska (2002d, g) did not accept Shoop's (1989) changes as she expected the systematics of the group to be more discernable once more data related to the morphology of cercaria and metacercaria is available. A more complete history of the Diplostomoidea was provided by Niewadomska (2002a–g). At the onset of the present studies, the Diplostomoidea included 6 families and 16 subfamilies: Bolbocephalodidae Strand, 1935 (no subfamily), Brauninidae Wolf, 1930 (no subfamily), Cyathocotylidae (5 subfamilies), Diplostomidae (4 subfamilies), Proterodiplostomidae (5 subfamilies) and Strigeidae (2 subfamilies) (Niewadomska, 2002a–g).

The current system of diplostomoidean families and subfamilies is based on a combination of adult and larval morphology and, in some cases, host associations (Niewiadomska, 2002a–g). Different authors referred to the two distinct body parts in diplostomoideans as prosoma + opisthosoma, forebody + hindbody or anterior + posterior segments. The most recent revision of the Diplostomoidea by Niewiadomska (2002a–g) in 'Keys to the Trematoda' utilized the terms forebody + hindbody. At the same time, a different meaning

was given to the same terms in chapters related to all other distome digeneans. We have opted to use the terms prosoma + opisthosoma, as forebody and hindbody are otherwise universally used to designate the part of the body anterior and posterior to the middle of the ventral sucker of distome digeneans. Furthermore, parts of the body in diplostomoideans are not segments (e.g., unlike segments of cestodes). Our use of this terminology is also consistent with its use in similar situations among other invertebrates, such as arachnids.

Members of the Cyathocotylidae and the Brauninidae can be differentiated from all other diplostomoideans by the presence of a cirrus sac and often have a unipartite body (Niewiadomska, 2002b, c; Achatz et al., 2019d). Whereas members of the Bolbocephalodidae, Diplostomidae, Proterodiplostomidae and Strigeidae lack a cirrus sac and typically have a bipartite body composed of a prosoma and opisthosoma. However, the prosoma and opisthosoma of some species may not be well-distinguished.

The body shape of non-cyathocotylid (and brauninid) diplostomoideans is often referred to as either ‘diplostomid’ or ‘strigeid’. The body shapes predominately differ in the structure of the prosoma (i.e., ‘diplostomid’ prosoma generally flattened vs ‘strigeid’ prosoma cup-like, tubular or bulbiform). Most members of the Diplostomidae and Proterodiplostomidae have a typical ‘diplostomid’ body, while most members of the Strigeidae and Bolbocephalodidae have a typical ‘strigeid’ body (e.g., Dubois, 1936a, b; Niewiadomska, 2002a–g; Achatz et al., 2019a).

Almost all members of the Diplostomidae and Proterodiplostomidae share a sucker-like holdfast organ located on the prosoma (Niewiadomska, 2002d, e; Tkach et al., 2020). In contrast, most members of the Strigeidae and Bolbocephalodidae have a bilobed holdfast organ positioned within the prosoma (Niewiadomska, 2002a, f). With only rare exceptions, members of the Proterodiplostomidae possess a paraprostate organ and are only known to parasitize reptiles as

adults (Niewiadomska, 2002e; Tkach et al., 2020). On the other hand, the members of the Diplostomidae, Strigeidae and Bolbocephalodidae lack a paraprostate organ (Niewadomska, 2002a, d, f; Blasco-Costa & Locke 2017).

The Diplostomoidea has been the focus of several molecular phylogenetic studies (e.g., Hernández-Mena et al., 2017; Locke et al., 2018; Achatz et al., 2019a–d; Achatz et al., 2020b; Queiroz et al., 2020; Tkach et al., 2020). Many of these molecular phylogenetic analyses have revealed the Diplostomidae and the Strigeidae to be non-monophyletic indicating the need for re-evaluation of these groups.

Aims and Objectives

Diplostomoidean parasites of a diversity of intermediate and definitive hosts were collected and analyzed to determine diversity of species, explore parasite-host relationships and explore evolutionary relationships among diplostomoideans. Diplostomoideans included in this study originate from every continent, except for Antartica, and every major invertebrate and vertebrate host lineage (e.g., mollusks, amphibians, fishes, reptiles, birds and mammals).

The aims and objectives of this study were:

1) To infer evolutionary relationships among major diplostomoidean lineages and provide a phylogenetic framework for future detailed molecular phylogenetic studies and a complete revision of the superfamily.
2) To analyze phylogenetic relationships within the Cyathocotylidae and use the molecular phylogeny to enhance the system of the family.

3) To analyze phylogenetic relationships within the Proterodiplostomidae and use the molecular phylogeny to enhance the system of the family.

4) To explore and describe the diversity of diplostomoidean taxa, including matching larval stages from intermediate hosts with adult forms from definitive hosts.

CHAPTER II

GENERAL MATERIALS & METHODS

Generalized methods are provided here to avoid repeating the same details in each chapter. Diplostomoideans collected in each study, alignment information and parameters of analyses are provided in the 'Materials & Methods' section of individual chapters. The abbreviations of genera names and authorities of taxa are used/provided separately within each chapter.

Morphological Methods

Adult diplostomoideans were removed from the intestines of a variety of definitive hosts. Parasites were briefly rinsed in saline. Live digeneans were heat killed with hot water and immediately fixed in 70% ethanol. Unless otherwise stated, dead digeneans were fixed immediately in 70% ethanol. The specimens for light microscopical study were stained with aqueous alum carmine (unless otherwise stated), dehydrated in ethanol series of ascending concentrations, cleared in clove oil and mounted permanently in Damar gum according to the protocol provided by Lutz et al. (2017). Diplostomoideans were measured with a DIC-equipped Olympus BX40 compound microscope (Tokyo, Japan) equipped with a digital imaging system. New species were drawn using a DIC-equipped Leica DMC 4500 microscope (Buffalo Grove,

Illinois, U.S.A.) with the aid of a drawing tube. Measurements provided in text are in micrometers unless otherwise noted.

Scanning electron microscopy (SEM) was conducted for some diplostomoideans. The specimens observed under SEM were dehydrated in an ethanol series of ascending concentrations and dried with hexamethyldisilazane (Ted Pella Inc., Redding, California, U.S.A.) as a transition fluid. Specimens were mounted on aluminum stubs using conductive double-sided tape, coated with gold-palladium and examined with the use of a Hitachi 4700 scanning electron microscope (Hitachi USA, Mountain View, California, U.S.A.) at an accelerating voltage of 5kV.

Molecular Methods

Entire or small fragments of specimens were used for molecular study, depending on the size of digeneans. Genomic DNA was extracted from the specimens following the protocol provided in Tkach & Pawlowski (1999) or ZR Genomic DNA™ Tissue Micro Prep kit (Zymo Research, Irvine, California, U.S.A.) following the manufacturer's protocol. Phylogenetic analyses were primarily based on a DNA fragment at the 5' end of the nuclear large ribosomal subunit gene (28S) gene. However, additional phylogenetic analyses of the ITS region (ITS1 + 5.8S + ITS2) rDNA and the cytochrome c oxidase 1 (*cox*1) mtDNA gene were performed as needed for different groups of diplostomoideans. Loci were amplified by polymerase chain reactions (PCR) using a T100^{TM} thermal cycler (Bio-Rad, Hercules, California, U.S.A.). Primers used in the studies are provided in Table 1. Polymerase chain reactions were performed in a total volume of 25 or 50 µl using New England Biolabs (Ipswich, Massachusetts, U.S.A.) OneTaq®

Table 1. Primers used for PCR and sequencing reactions of the large ribosomal subunit (28S) and ITS region (ITS1 + 5.8S + ITS2) rDNA and cytochrome c oxidase 1 (*cox*1) mtDNA loci.

Primer	Sequence	Locus	Reference
1500R	5'–GCT ATC CTG AGG GAA ACT TCG–3'	28S	Tkach et al., 2003
digL2	5'–AAG CAT ATC ACT AAG CGG–3'	28S	Tkach et al., 2003
DPL600F	5'–CGG AGT GGT CAC CAC GAC CG–3'	28S	Achatz et al., 2019d
DPL700R	5'–CAG CTG ATT ACA CCC AAA G–3'	28S	Achatz et al., 2019d
LSU	5'–TAG GTC GAC CCG CTG AAY TTA AGC A–3'	28S	Olson et al. 2003
300R	5'– CAA CTT TCC CTC ACG GTA CTT G –3'	ITS/28S	Snyder & Tkach, 2007
d58F	5'– GCG GTG GAT CAC TCG GCT CGT G –3'	ITS	Kudlai et al., 2015
ITSf	5'– CGC CCG TCG CTA CTA CCG ATT G –3'	ITS	Snyder & Tkach, 2007
Plat-diploCOX1F	5'–CGT TTR AAT TAT ACG GAT CC–3'	*cox*1	Moszczynska et al., 2009
Cox1_Schist_5'	5'–TCT TTR GAT CAT AAG CG–3'	*cox*1	Lockyer et al., 2003
Plat-diploCOX1R	5'–AGC ATA GTA ATM GCA GCA GC–3'	*cox*1	Moszczynska et al., 2009
JB5	5'–AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG–3'	*cox*1	Derycke et al., 2005
BS_CO1_IntF	5'–ATT AAC CCT CAC TAA ATG ATT TTT TTY TTT YTR ATG CC–3'	*cox*1	Achatz et al., 2019a, c
BS_CO1_IntR	5'–TAA TAC GAC TCA CTA TAA AAA AAA MAM AGA AGA RAA MAC MGT AGT AAT–3	*cox*1	Achatz et al., 2019a, c
Dipl_Cox_5'	5'–ACK TTR GAW CAT AAG CG–3'	*cox*1	Achatz et al. *in review*
Dipl_Cox_3'	5'–WAR TGC ATN GGA AAA AAA CA–3'	*cox*1	Achatz et al. *in review*
Dipl650R	5'–CCA AAR AAY CAR AAY AWR TGY TG–3'	*cox*1	Achatz et al. *in review*

Quick-Load® 2X Master Mix polymerase or GoTaq® G2 DNA polymerase from Promega (Madison, Wisconsin, U.S.A.) according to the manufacturers' protocol. The annealing temperatures were typically 53ºC for rDNA reactions and 45ºC for *cox*1 reactions.

ExoSAP-IT PCR clean-up enzymatic kit from Affymetrix (Santa Clara, California, U.S.A.) and Illustra ExoProStar PCR clean-up enzymatic kit from Cytiva (Marlborough, Massachusetts, U.S.A.) were used to purify PCR products. MCLab BrightDye® terminator chemistry (Molecular Cloning Laboratories, San Francisco, California, U.S.A.) was used to

cycle-sequence PCR products with PCR primers (Table 1). Sequencing reactions were subsequently cleaned using MCLab BigDye magnetic beads and run on an ABI 3130 automated capillary sequencer (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.). Contiguous sequences were assembled using Sequencher ver. 4.2 (GeneCodes Corp., Ann Arbor, Michigan, U.S.A.) and submitted to GenBank. Each chapter contains the relevant GenBank accession numbers.

CHAPTER III

MOLECULAR PHYLOGENY OF THE CYATHOCOTYLIDAE (DIGENEA, DIPLOSTOMOIDEA) NECESSITATES SYSTEMATIC CHANGES AND REVEALS A HISTORY OF HOST AND ENVIRONMENT SWITCHES

Introduction

The Cyathocotylidae Mühling, 1898 is a small, globally distributed family of diplostomoidean digeneans that are parasitic as adults in the intestine of birds, reptiles and, rarely, mammals and fishes. Unlike most other diplostomoideans, cyathocotylids usually have an undivided body, a cirrus sac enclosing a cirrus and a seminal vesicle and, sometimes, a small caudal appendix (Niewiadomska, 2002c). Some members of this family are of economic and conservation concern; for instance, *Cyathocotyle bushiensis* Khan, 1962 has been associated with massive die-offs of aquatic birds in the Midwestern United States (Gibson et al., 1972; Hoeve & Scott, 1988; Herrmann & Sorensen, 2009). Mühling (1896) initially established the subfamily Cyathocotyleae Mühling, 1896 for his newly described genus *Cyathocotyle* Mühling, 1896 with *Cyathocotyle prussica* Mühling, 1896 as the type-species. Later, Poche (1926) elevated the status of the group to family level. The most recent revision of the Cyathocotylidae by Niewiadomska (2002c) recognized five subfamilies: Cyathocotylinae Mühling, 1896 (four genera), Muhlinginae Mehra, 1950 (one genus), Prohemistominae Lutz, 1935 (five genera), Prosostephaninae Szidat, 1936 (three genera) and Szidatiinae Dubois, 1938 (three genera).

The current systematics and taxonomy of the Cyathocotylidae is based entirely on morphological characters. While members of the family parasitize fishes, reptiles, birds and mammals worldwide, the lack of a robust (or any) phylogeny of the group prevented addressing intriguing questions regarding patterns of their current and past geographic distribution, host associations and environmental switches. In fact, no molecular phylogenetic study of the family has been carried out. Monophyly of the family as a whole and its constituent subfamilies and genera have not been tested yet using molecular data. Likewise, the interrelationships among the genera within the Cyathocotylidae remain completely unknown. Currently, mostly non-comparable DNA sequences are available from the adult forms of only three species belonging to the genera, *Mesostephanus* Lutz, 1935 and *Holostephanus* Szidat, 1936 (Dzikowski et al., 2004; Hernández-Mena et al., 2017; El-Bahy et al., 2017); moreover, all three species are from avian hosts, thus preventing evolutionary analysis of the patterns of host associations among cyathocotylids.

Other studies have generated DNA sequence data from other life cycle stages, mostly cercariae and metacercariae (Locke et al., 2010; Karamian et al., 2011; Ciparis et al., 2013; Blasco-Costa & Locke, 2017; Locke et al., 2018); however, the limited sequence data from adult forms prevent accurate species- or genus-level diagnoses of the sequenced life cycle stages. While recent molecular phylogenetic studies (Blasco-Costa & Locke, 2017; Hernández-Mena et al., 2017; Locke et al., 2018) have indicated the general position of the Cyathocotylidae among other diplostomoidean families, the very limited number of taxa used in these analyses did not allow exploration of questions of evolution and systematics related to geographic distribution, host associations and environmental switches of the group. For instance, no molecular data have been published on any of the genera of cyathocotylids parasitizing non-avian reptiles and fish as

adults. From a geographic point of view, all available cyathocotylid DNA sequences so far come from Europe and North America, thus leaving their relationships with members of the family from other regions completely unknown.

Similarly, the phylogenetic affinities of another diplostomoidean family, the Brauninidae Wolf, 1903, have always been unclear. At present, the Brauninidae only includes the monotypic genus *Braunina* Heider, 1900 that parasitize cetaceans as adults. *Braunina* shares atypical diplostomoidean morphological characters with the Cyathocotylidae (e.g. a cirrus sac enclosing a cirrus and a seminal vesicle) (Niewiadomska, 2002b). The validity and systematic position of the Brauninidae in relation to the Cyathocotylidae has been recently called into question based on the results of molecular phylogenetic studies of the group (Fraija-Fernández et al. 2015; Blasco-Costa & Locke, 2017; Hernández-Mena et al., 2017) which placed Braunina in the clade with members of the Cyathocotylidae. However, the Brauninidae currently remains a separate family due to insufficient amount of data and low diversity of cyathocotylids included in previous phylogenetic analyses.

Herein, we examine the phylogenetic interrelationships and host associations of the Cyathocotylidae and re-evaluate the taxonomic status of its constituting lineages as well as the family Brauninidae using 28S rRNA gene sequences from quality specimens of cyathocotylids newly collected from fish, reptiles, birds and mammals in Europe, Asia, Australia, Africa and North America. In addition, we used mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) gene sequences for comparison between samples of *Braunina* originating from different hosts and geographic regions as well as between two genetically different forms identified as *Mesostephanus microbursa* Caballero, Grocott et Zerecero, 1953.

Materials & Methods

Specimens

Specimens belonging to the families Cyathocotylidae and Brauninidae were collected from the intestines of fish, snakes, crocodilians, birds and dolphins in Australia, Southeast Asia, Europe and North America (Table 2). The digeneans from the Nile crocodile *Crocodylus niloticus* Laurenti were killed in hot saline, fixed in 10% formalin and transferred to 70% ethanol. Morphological vouchers are deposited in the collection of the Harold W. Manter, University of Nebraska State Museum, Lincoln, NE, U.S.A.

Phylogenetic analyses

Sequences were initially aligned using ClustalW implemented in MEGA7 (Kumar et al., 2016). The alignment was then trimmed to the length of the shortest sequences. *Clinostomum tataxumui* Sereno-Uribe, Pinacho-Pinacho, Garcia-Varela et Pérez-Ponce de León, 2013 was used as outgroup based on the phylogeny published by Hernández-Mena et al. (2017). The alignment included newly obtained sequences of one specimen of *Braunina* sp. and 10 cyathocotylid taxa, previously published sequences of *Braunina coridiformis*, three cyathocotylid taxa, 13 representatives of the Diplostomidae Poirier, 1886, one species of the Proterodiplostomidae Dubois, 1936 and 10 taxa of the Strigeidae Railliet, 1919 in order to test the interrelationships among all these digenean families.

Phylogenetic analyses were conducted using Bayesian inference (BI) as implemented in MrBayes Ver. 3.2.6 software (Ronquist & Huelsenbeck, 2003) and Maximum Likelihood (ML) as implemented in MEGA7 (Kumar et al., 2016). The general time reversible model with estimates of invariant sites and gamma distributed among-site variation (GTR + I + G) was

Table 2. List of cyathocotylid species sequenced including their host species, geographical origin of material, morphological voucher numbers and GenBank accession numbers. HWML: Harold W. Manter Laboratory, University of Nebraska State Museum, Lincoln, NE, U.S.A.

Digenean taxa	Host species	Country	Museum No.	Accession numbers	
				28S	***cox*1**
Braunina sp.	*Tursiops truncatus*	U.S.A.	HWML-110857	MK650438, MK650439	MK645805
Cyathocotyle bushiensis	*Aythya affinis*	U.S.A.	HWML-139967	MK650440	–
Gogatea mehri	*Xenochrophis flavipunctatus*	Vietnam	–	MK650441	–
Gogatea sp.	*Acrochordus javanicus*	Thailand	–	MK650442	–
Holostephanoides ictaluri	*Ameiurus* sp.	U.S.A.	_	MK650443	–
Holostephanus dubinini	*Phalacrocorax carbo*	Ukraine	HWML-139968	MK650444	–
Mesostephanus cubaensis	*Morus bassanus*	U.S.A.	HWML-139969	MK650445	–
Mesostephanus microbursa	*Mo. bassanus*	U.S.A.	HWML-139970	MK650446	MK645806
Neogogatea sp.	*Lophodytes cucullatus*	U.S.A.	HWML-139971	MK650447–MK650449	–
Suchocyathocotyle crocodili	*Crocodylus johnstoni*	Australia	HWML-139972	MK650450, MK650451	–
Suchocyathocotyle fraterna	*Crocodylus niloticus*	South Africa	HWML-139373	MK650452	–

identified as the best-fitting nucleotide substitution model using jModelTest 2 software (Darriba et al., 2012). Nodal support of ML analysis was estimated by performing 1000 bootstrap pseudoreplicates. BI analysis was performed using MrBayes software as follows: Markov chain Monte Carlo (MCMC) chains were run for 3,000,000 generations with sample frequency set at 100. Log-likelihood scores were plotted and only the final 75% of trees were used to produce the consensus trees by setting the "burn-in" parameter at 7,500. This number of generations was considered sufficient because the SD dropped below 0.01.

Results

The 28S rRNA alignment was 1,083 bp long. In the phylogenetic trees resulting from the BI and ML analyses, all members of the Cyathocotylidae and Brauninidae formed a very highly supported (100% in BI and 99% in ML), monophyletic clade (Fig. 1). Within this clade, *Cyathocotyle* (*Suchocyathocotyle*) *crocodili* from the freshwater crocodile *Crocodylus johnsoni* Krefft collected in Australia, and *Cyathocotyle* (*Suchocyathocotyle*) *fraterna* collected from the Nile crocodile *Crocodylus niloticus* in South Africa formed a very highly supported clade (100% in BI and 99% in ML) as the sister-group to the remaining cyathocotylid taxa. All other cyathocotylids included in our analysis formed the second, very highly supported clade (100% in BI and ML) with overall highly supported internal topology. One of the subclades included strongly supported clusters of *Cyathocotyle* + *Holostephanus* (100% in BI and ML) and *Gogatea* Lutz, 1935 + *Neogogatea* Chandler et Rausch, 1947 + *Holostephanoides* Dubois, 1983 (100% in BI and 97% in ML). Both sequenced forms of *Braunina* were nested within the Cyathocotylidae clade and formed a strongly supported clade with the *Mesostephanus* spp. and

an unknown cyathocotylid cercaria from Australia (98% in BI and 93% in ML). All three adult forms of *Mesostephanus* formed a strongly supported clade (100% in BI and ML) (Fig. 1).

At the species level, our DNA sequences obtained from a *Braunina* specimen collected from *Tu. truncatus* in the Gulf of Mexico differed by two bases (0.16%) in 28S and by 41 bases (8.9%) in *cox*1 from the comparable sequences of *Br. cordiformis* from *Delphinus delphis* Linnaeus available in GenBank (KM258670, MF124272). The phylogenetic analysis indicated that our 28S sequence of *Me. microbursa* does not form a monophyletic group with the sequence of *Me. microbursa* from GenBank (MF398325). However, Hernández-Mena et al. (2017) have also published a *cox*1sequence from the same specimen (GenBank MF398316). We, therefore, also obtained *cox*1sequence from our sample for comparison. The two forms differed by 30 nucleotide positions (2.7%) in 28S sequences and 80 nucleotide positions (16.4%) in *cox*1sequences.

Other accepted families of the superfamily Diplostomoidea formed a strongly supported (100% in BI and ML) clade (Fig. 1). This clade was, however, poorly resolved internally with multiple taxa of the Diplostomidae, Proterodiplostomidae and Strigeidae forming a polytomy. The Strigeidae as currently accepted was polyphyletic with two distinct clades. The first clade was strongly supported (100% in both BI and ML) and included members of *Apatemon* Szidat, 1928, *Australapatemon* Sudarikov, 1959 and a well-supported group of *Strigea* Abildgaard, 1790 + *Apharyngostrigea* Ciurea, 1927 + *Parastrigea* Szidat, 1928. The second supported clade (97% in BI and 62% in ML) united *Cardiocephaloides* Sudarikov, 1959 and a strongly supported clade of *Cotylurus* Szidat, 1928 + *Ichthyocotylurus* Odening, 1969 (100% in BI and 99% in ML).

The Diplostomidae were also found to be paraphyletic and formed seven distinct clades: 1) *Posthodiplostomum* Dubois, 1936 + *Ornithodiplostomum* Dubois, 1936 (100% in both BI and

ML), 2) *Bolbophorus* Dubois, 1935, 3) *Uvulifer* Yamaguti, 1934, 4) *Diplostomum* von Nordmann, 1832 + *Austrodiplostomum* Szidat et Nani, 1951 + *Tylodelphys* Diesing, 1850 (100% in BI and 91% in ML), 5) *Alaria* Schrank, 1788, 6) *Neodiplostomum* Railliet, 1919 and 7) *Hysteromorpha* Lutz, 1931.

Discussion

The Cyathocotylidae is a relatively small digenean group, therefore, even a limited set of taxa allowed us to answer some questions about their systematics and reveal important trends in the evolution of their morphological traits, host associations and geographic distribution. We have for the first time obtained and combined in one study sequences of cyathocotylids from five continents and all main host groups (fishes, snakes, crocodilians, birds and mammals). The results of our phylogenetic analysis challenge the current morphology-based systematic framework of the Cyathocotylidae in several ways. While the molecular phylogeny supported the monophyly of some of the existing subfamilies (Fig. 1 and discussion below), the paraphyletic nature of the type-genus of the family as well as the position of *Holostephanoides* outside of the Cyathocotylinae and particularly the inclusion of *Braunina* within the Cyathocotylidae, necessitates taxonomic and systematic changes. Below we discuss the main findings of this study focusing on well-supported topologies only.

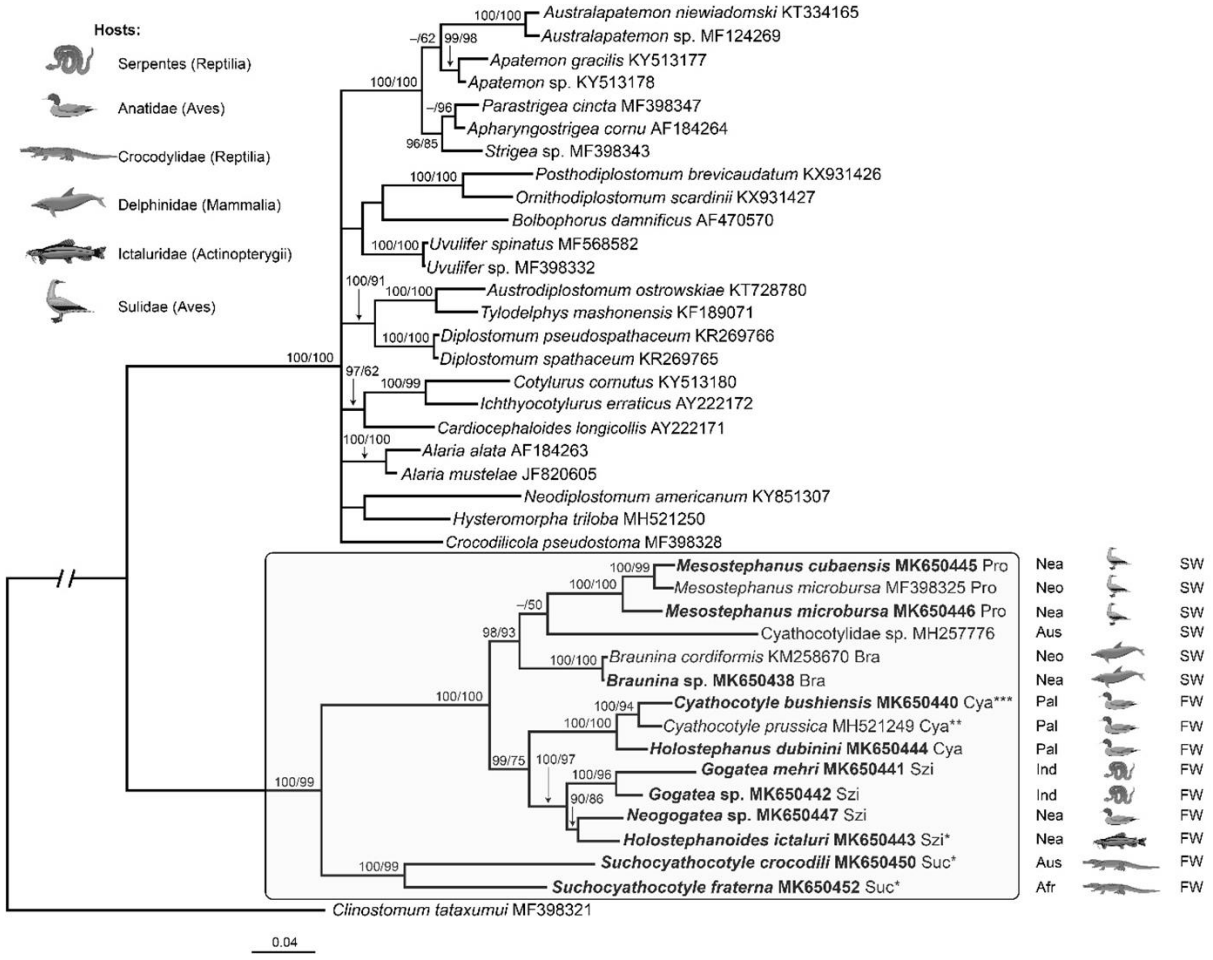

Figure 1. Molecular phylogeny of the Diplostomoidea with a focus on the Cyathocotylidae based on Bayesian inference (BI) and Maximum Likelihood (ML) analyses of partial 28S rRNA gene sequences. Topology from BI analysis provided. Bayesian inference posterior probability values and Maximum Likelihood bootstrap values associated with the branches are shown as BI/ML; support values lower than 90% (BI) and 50% (ML) are not shown. The gray box surrounds members of the Cyathocotylidae. New sequences obtained in this study are in bold. Branch length scale bar indicates number of substitutions per site. GenBank accession numbers are provided after the names of species. Biogeographical realms, definitive host groups and types of environment, and subfamilies are indicated for the members of the Cyathocotylidae only. Abbreviations for biogeographical realms: Afr, Afrotropical realm; Aus, Australasian realm; Ind, Indo-Malayan realm; Nea, Nearctic realm; Neo, Neotropical realm; Pal, Palearctic realm. Abbreviations for cyathocotylid subfamilies: Bra, Brauninininae; Cya, Cyathocotylinae; Pro, Prohemistominae; Suc, Suchocyathocotylinae; Szi, Szidatiinae. Abbreviations for environment type of definitive host: FW, freshwater; SW, saltwater. *Previously a member of subfamily Cyathocotylinae. **The definitive host of *C. prussica* is a member of Anatidae, but the sequence was obtained from a metacercaria collected from fish. ****Cyathocotyle bushiensis* is a Palearctic species only recently introduced to the Nearctic. (after Achatz et al., 2019d)

Braunina and the amended diagnosis of the Cyathocotylidae

The genus *Braunina* is a unique group of diplostomoideans parasitizing cetaceans as adults and is the only genus of the family Brauninidae (Niewiadomska, 2002b). *Braunina* was first included in a molecular phylogenetic analysis by Fraija-Fernández et al. (2015), however, no cyathocotylids were included in their analysis. Recent studies by Hernández-Mena et al. (2017) and Blasco-Costa & Locke (2017) called the systematic position of the Brauninidae in relation to the Cyathocotylidae into question. Blasco-Costa & Locke (2017) demonstrated that *Br. cordiformis* forms a clade with a metacercaria of unknown species of *Cyathocotyle* (the name was later changed to *Cy. prussica*) and *Mesostephanus* based on *cox*1 mtDNA and ITS region rDNA sequences. Both Hernández-Mena et al. (2017) and Blasco-Costa & Locke (2017) have noted the strong morphological similarities between *Braunina* and cyathocotylids and their differences from other diplostomoideans (e.g. presence of a cirrus sac in *Braunina* and cyathocotylids vs absence in all other diplostomoideans). While Blasco-Costa & Locke (2017) indicated that the Cyathocotylidae would be monophyletic upon inclusion of the Brauninidae, no definitive taxonomic conclusion was drawn. Those authors stated that knowledge of the morphology of the larval stages of *Braunina* would eventually strengthen the argument for its transfer to the Cyathocotylidae. In our opinion, larval morphology is desirable, but not required for this taxonomic action. Our molecular phylogenetic results as well as the details of adult morphology provide sufficient grounds for synonymization. Therefore, we transfer *Braunina* into the family Cyathocotylidae and the Brauninidae becomes a junior synonym of the Cyathocotylidae. It is noteworthy that the Brauninidae had already been considered a synonym of the Cyathocotylidae in the past (Wolf, 1903; La Rue, 1957; Yamaguti, 1971). At the same time, based on its unique morphology and the results of our phylogenetic analysis, we retain this

taxon as subfamily Braunininae Wolf, 1903 within the Cyathocotylidae, with diagnosis of the type- and currently only genus *Braunina*. The amended diagnosis of the Cyathocotylidae is provided below.

Cyathocotylidae Mühling, 1896.

Diagnosis (after Niewiadomska, 2002c, with changes): Diplostomoidea, with body generally undivided, oval, pyriform, linguiform, or cordiform, with small, conical, truncate, or sometimes elongate caudal appendix. Holdfast organ round or oval, sometimes large and overlaid by ventral fold forming deep cavity (in *Braunina*). Oral sucker present or absent; when absent, subterminal oral opening leads directly to pharynx. Ventral sucker present or absent. Pseudosuckers absent. Oesophagus short; caeca usually reaching close to posterior end of body, rarely sinuous. Position of testes and ovary variable. Cirrus sac present, occasionally rudimentary, enclosing seminal vesicle, pars prostatica, and cirrus. Genital pore terminal. Eggs typically large, not numerous. Vitellarium follicular, variable in extent. Life-cycle with longifurcate furcocercous cercaria having excretory system composed of four stems united anteriorly, two lateral and two median joined anterior to ventral sucker. Metacercaria of 'prohemistomulum' type, with sucker-like holdfast organ and crown-like reserve bladder. Parasites of reptiles, birds, and mammals. Mother- and daughter-sporocysts in gastropods (Prosobranchia); metacercariae in fishes, amphibians, and aquatic invertebrates. Type-genus *Cyathocotyle* Mühling, 1896.

Braunina contains the only valid species *Br. cordiformis* originally described from the short-beaked common dolphin *De. delphis* collected in the Adriatic Sea (Wolf, 1903). Previously published sequences of *Br. cordiformis* (GenBank KM258670, MF124272) also came from material obtained from a *De. delphis*, but collected off the Atlantic coast of Argentina (Fraija-

Fernandez et al., 2015). Our DNA sequences obtained from a *Braunina* species collected from *Tu. truncatus* in the Gulf of Mexico differ by 8.9% in *cox*1 from the matching sequence of *Br. cordiformis* in GenBank, thus strongly indicating the presence of a second species in the genus. At the same time, relationships of either of these species with the form originally described from the Adriatic Sea remain unknown. Molecular data for *Br. cordiformis* collected close to the type-locality, are needed to verify the identity of currently available sequences.

It is possible that *Br. cordiformis* is distributed in both the Old and New World, however, it may also be a complex of more than two morphologically similar species. While our specimens appear in relatively fair condition, they are not ideal for descriptive work, especially considering their size and shape. Due to the protected status of dolphins, opportunities to obtain fresh material from them are very rare and chances of obtaining live digeneans that can be properly fixed are extremely low. Until quality specimens associated with DNA sequences become available, knowledge of the diversity of the genus will mostly rely on molecular data.

Composition of the Cyathocotylinae Mühling 1896

The most recent revision of the Cyathocotylinae by Niewiadomska (2002c) recognized four genera: *Cyathocotyle*, *Holostephanoides*, *Pseudhemistomum* Szidat, 1936 and *Holostephanus*. Our analysis included three of these genera (*Cyathocotyle*, *Holostephanus* and *Holostephanoides*). According to the most recent revision of *Cyathocotyle* by Dubois (1984), this genus contains seventeen species distributed on all continents except for Antarctica and Australia. Fourteen species are parasites of birds as adults, while the remaining three species have been described from crocodilians. No *Cyathocotyle* species has been described in North America, however, the European species *Cy. bushiensis* was reported from the Midwestern

United States. Often, these reports were associated with massive die-offs of waterfowl (Gibson et al., 1972; Hoeve & Scott, 1988; Herrmann & Sorensen, 2009). Surprisingly, no DNA sequences of this rather infamous species were available until now.

Originally, *Cy. bushiensis* was described in the United Kingdom based on digeneans experimentally grown in laboratory ducklings from metacercariae obtained from naturally infected prosobranch snails belonging to *Bithynia tentaculata* Linnaeus (Khan, 1962). The first study of *Cy. bushiensis* in North America compared the morphology of North American and European specimens and noted several morphological differences between them including egg length, relative cirrus length, shape and relative size of testes (Gibson et al., 1972). Our specimens collected from Lake Winnibigoshish, Minnesota very closely correspond to those described by Khan (1962).

Currently the genus *Cyathocotyle* is split into the subgenera *Cyathocotyle* Mühling, 1896 and *Suchocyathocotyle* Dubois, 1984 on the basis of testes orientation (opposite testes in *Cyathocotyle* and tandem in *Suchocyathocotyle*), cirrus sac length (reaching or extending beyond the middle of the body in *Cyathocotyle* and never extending beyond the middle of the body in *Suchocyathocotyle*), egg size (smaller in *Cyathocotyle* and larger in *Suchocyathocotyle*), and definitive hosts (birds in *Cyathocotyle* and crocodilians in *Suchocyathocotyle*). It should be noted that the relative length of the cirrus sac cannot be used for reliable differentiation between these taxa because at least some species of *Cyathocotyle* (*Cyathocotyle*) have a short cirrus sac not fitting Dubois' (1984) diagnosis. On the other hand, one species of *Cyathocotyle* (*Cyathocotyle*), namely *Cyathocotyle* (*Cyathocotyle*) *fulicae* Ginetzinskaja, 1952, was described as having tandem testes. However, it is evident from the provided illustration (Ginetzinskaja, 1952) that the

specimen was very strongly flattened at the time of fixation which likely caused a shift in the position of internal organs.

Suchocyathocotyle was named after the crocodilian hosts of three of the four included species: *Cy.* (*S.*) *crocodili* (type-species) was described from the saltwater crocodile *Crocodylus porosus* Schneider in Indonesia, *Cyathocotyle* (*Suchocyathocotyle*) *brasiliensis* Ruiz et Leão, 1943 from the spectacled caiman *Caiman crocodilus* Linnaeus (=*Caiman sclerops*) in Brazil, *Cy.* (*S.*) *fraterna* from *Cr. niloticus* (=*Champse vulgaris*) in Egypt (Odhner, 1902; Ruiz & Leão, 1943; Yamaguti, 1954; Dubois, 1984). The fourth species, *Cyathocotyle* (*Suchocyathocotyle*) *szidatiana* Faust et Tang, 1938, was described from a mallard duck *Anas platyrhynchos* Linnaeus (=*Anas boschas*) in China (Faust & Tang, 1938). Dubois (1984) argued that the duck infection with *Cy.* (*S.*) *szidatiana* may have been accidental and the true definitive host is a crocodilian, presumably the Chinese alligator *Alligator sinensis* Fauvel.

Our phylogenetic analysis places the type-species *Cy.* (*S.*) *crocodili* and *Cy.* (*S.*) *fraterna* as the basal branch within the Cyathocotylidae, genetically distant from *Cyathocotyle* parasitic in birds. Based on the results of our molecular phylogenetic analysis combined with the morphological characters used by Dubois (1984) to separate the subgenera of *Cyathocotyle*, we elevate these two subgenera to genus status. Therefore, *Cy.* (*S.*) *crocodili* (type-species), *Cy.* (*S.*) *brasiliensis*, *Cy.* (*S.*) *fraterna* and *Cy. (S.) szidatiana* are transferred to *Suchocyathocotyle* Dubois, 1984. However, we place the latter species in *Suchocyathocotyle* with caution. Although it has tandem testes, the description of some important morphological characters in this species is vague. In general, multiple cases of poor descriptions and/or poor fixation of specimens used for descriptions in the literature present a major hinderance for a full revision of the group. Collection of fresh, properly fixed specimens is desirable for the majority of species. For

instance, the cirrus sac of *S. szidatiana* as described by Faust & Tang (1938) was strongly contracted, which causes uncertainty and places it between *Cyathocotyle* and *Suchocyathocotyle*. Thus, the systematic position of *S. szidatiana* may eventually change. Due to the fact that the diagnoses of the subgenera *Cyathocotyle* and *Suchocyathocotyle* by Dubois (1984) were very brief and Niewiadomska's (2002c) diagnosis combined features of both Dubois' subgenera, there is an obvious need for amended diagnoses of *Cyathocotyle* and *Suchocyathocotyle*. They are provided below. We would like to emphasize that only properly mounted, not flattened specimens are likely to fit these diagnoses. Also, the only species of *Cyathocotyle* (*Cyathocotyle*) with eggs larger than 113 µm is *Cyathocotyle* (*Cyathocotyle*) *melanittae* Yamaguti, 1934, in which the position of testes is uncertain, appearing nearly tandem in a flattened, laterally mounted specimen (Yamaguti, 1934).

Cyathocotyle Mühling, 1896.

Diagnosis (after Dubois, 1984 and Niewiadomska, 2002c, with changes): Body massive, oval, pyriform or fusiform. Holdfast organ large, round, with aperture of variable shape, elevated above ventral surface. Oral sucker and pharynx well-developed; ventral sucker small, near intestinal bifurcation, in some species absent or not visible, covered by holdfast organ. Oesophagus very short or absent. Testes round or elongate, opposite or somewhat oblique. Cirrus sac well-developed, claviform, with large seminal vesicle occupying proximal part of cirrus sac. Genital pore subterminal. Ovary round, small, variable in position and ventral to testes. Eggs small to medium sized (57–127µm). Vitellarium in form of coarse follicles surrounding holdfast organ in peripheral part of body and overlying caeca, usually does not extend into organ. In different groups of birds. Europe, Asia, North America. Metacercariae of 'prohemistomulum'

type, in fishes or leeches; one species in *Bithynia* Leach. Cercariae, with flame-cell formula 2[(3 + 3) + (3 + [3])] = 24, developing in Prosobranchia (*Bithynia*, *Bellamya* Jousseaume) or Pulmonata (*Bulinus* Müller). Type-species *Cy. prussica* Mühling, 1896. Other species: *Cy. anhinga* Vidyarthi, 1948, *Cy. bithyniae* Sudarikov, 1974, *Cy. bushiensis* Khan, 1962, *Cy. fulicae* Ginetzinskaja, 1952, *Cy. indica* Mehra, 1943, *Cy. japonica* Kurisu, 1931, *Cy. malayi* Palmieri, Krishnasamy et Sullivan, 1979, *Cy. melanittae*, *Cy. opaca* (Wisniewski, 1934), *Cy. orientalis* Faust, 1922, *Cy. oviformis* Szidat, 1936, *Cy. skrjabini* Petrov et Sudarikov, 1963.

Suchocyathocotyle Dubois, 1984.

Diagnosis (after Dubois, 1984, with changes): Body massive, oval, pyriform with or without caudal appendix. Holdfast organ large, round, with aperture of variable shape, elevated above ventral surface. Oral sucker and pharynx well-developed; ventral sucker weakly developed, near intestinal bifurcation. Prepharynx and oesophagus very short. Ceca reaching to second or last third of body. Testes oval, tandem. Cirrus sac claviform, thin-walled, typically short, never extending beyond equator of body, containing elongated seminal vesicle, terminating with muscular ejaculatory duct. Genital pore subterminal. Ovary round, small, either at the level of the anterior testis or inter-testicular. Uterus with few coils, metraterm short. Laurer's canal absent. Eggs large sized (117 to 144 μm). Vitellarium in form of large coarse follicles, filling most of body. Excretory pore ventral to genital pore; dorso-lateral stems reach as far as anterior extremity. Parasites of crocodilians in Africa, Asia, Australia and South America. Type-species: *S. crocodili* (Yamaguti, 1954). Other species: *S. fraterna* (Odhner, 1902), *S. brasiliensis* (Ruiz et Leão, 1943), *S. szidatiana* (Faust et Tang, 1938).

Based on the results of the phylogenetic analysis demonstrating that *Suchocyathocotyle* forms a clade clearly separated from Cyathocotylinae and the remaining cyathocotylid subfamilies included in our analysis, we establish herein a subfamily Suchocyatocotylinae subfam. n. with *Suchocyathocotyle* as the type- and currently only genus. The diagnosis of the subfamily is the same as that of *Suchocyathocotyle*.

The two *Suchocyathocotyle* sequences included in our analysis are characterized by long branches which likely reflects the long evolutionary and geographic separation between these parasites and their hosts. While it is possible that the African species *S. fraterna* may deserve to be separated into its own genus, the morphological study of our specimens as well as descriptions in the literature did not yield convincing evidence to warrant creation of a new genus.

Composition of the Szidatiinae Dubois, 1938

The most recent revision of the subfamily Szidatiinae included the three genera: *Szidatia* Dubois, 1938, *Gogatea* and *Neogogatea* (Niewiadomska, 2002c). Adult *Gogatea* and *Szidatia* parasitize snakes in Africa and Asia (Joyeux & Bear, 1934; Gogate, 1932), whereas *Neogogatea* parasitizes birds in North America and Asia (Chandler & Rausch, 1947; Zazornova, 1995). No member of the subfamily has been used in any molecular study to date. Our analysis used two of the genera (*Gogatea* and *Neogogatea*) and confirmed their close relationships and separation from other main cyathocotylid linages (Fig. 1).

Dubois (1989) recognized *Gogatea serpentum* (Gogate, 1932) as the only member of *Gogatea* with two subspecies, *Gogatea serpentum serpentum* (Gogate, 1932) and *Gogatea. serpentum indicum* Mehra, 1947. The latter form had been elevated to species level by Dwivedi

& Chauhan (1969), based on several morphological characteristics (including the relative length of cirrus sac and position of testes), and named *Gogatea mehri* Mehra, 1947. Dubois (1975; 1980) rejected this change and synonymized *G. mehri* as well as three other species, with *G. serpentum indicum*. Our, high quality specimens, freshly collected in Vietnam, fully corresponded morphologically to the description of *G. mehri*, but not to the original description of *G. serpentum*. Therefore, we consider *G. mehri* a separate species. The taxonomic status of other species synonymized by Dubois (1975; 1980) requires a revision using quality specimens.

The present work is the first molecular systematic study to include sequences of any member of either *Gogatea* or *Neogogatea*. Our specimens of *Neogogatea* sp. (deposited as HWML-139971), collected from the hooded merganser *Lophodytes cucullatus* (Linnaeus) in Mississippi, U.S.A., were not fully mature (lacked eggs), but still had traits of *Neogogatea* (e.g. lack of ventral sucker and vitellarium in form of horseshoe).

Somewhat surprisingly, *Holostephanoides ictaluri* Vernberg, 1952, the type-species of *Holostephanoides* (previously a member of the Cyathocotylinae), appeared in the phylogenetic tree within a strongly supported clade with members of Szidatiinae with *Neogogatea* as its closest relative. While the general morphology of *Holostephanoides* (a digenean with rounded appearance), *Gogatea* and *Neogogatea* (both include digeneans with an enlarged anterior part of the body and elongated posterior part) differs substantially, the phylogenetic analysis suggests that these differences are likely a result of recent adaptation. Other than the body shape, these genera do not have other dramatic morphological differences. Moreover, available data on the excretory system including the protonephridial formulas support relatedness between *Holostephanoides* and *Neogogatea*. Both genera have the same excretory formula 2[(3+3+3)+(3+3+[3])]=36 (Cable, 1938; Hoffman & Dunbar, 1963; Stang & Cable, 1966). On

the other hand, *Cyathocotyle* and *Holostephanus* belonging to the Cyathocotylinae have different protonephridial formulas, 2[(3+3)+3)3+[3])]=24 in *Cyathocotyle* and 2[(2+2+2)+(2+2+[2])]=24 in *Holostephanus* (Dubois, 1983; Dubois, 1984). The excretory formula of *Pseudhemistomum*, the other genus belonging to subfamily Cyathocotylinae, is currently unknown. Due to the fact that our analysis included the type-species of the genus, we transfer *Holostephanoides* into the Szidatiinae. However, the monophyly of *Holostephanoides* needs to be tested using DNA sequences and data on the excretory system of its only other member, *Holostephanoides hoeppliana* (Tang & Tang, 1989) from Eurasian curlew *Numenius arquata* (Linnaeus) in China.

Notes on the Prohemistominae Lutz, 1935

The most recent revision of subfamily Prohemistominae by Niewiadomska (2002c) includes five genera: *Mesostephanoides* Dubois, 1951, *Mesostephanus*, *Prohemistomum* Odhner, 1913, *Linstowiella* Szidat, 1933 and *Paracoenogonimus* Katsurada, 1914. Our analyses are limited to *Mesostephanus*, a cosmopolitan genus of cyathocotylids that parasitize birds and mammals as adults. Our analysis shows strong support for the *Mesostephanus* clade.

We compared our sequences of *Me. microbursa* collected from the Northern gannet *Morus bassanus* (Linnaeus) off the coast of Mississippi with the sequence of *Me. microbursa* (GenBank MF398316, MF398325) collected from the blue-footed booby *Sula nebouxii* Milne-Edwards in Nayarit, Mexico (Hernández-Mena et al., 2017). These samples differed by 2.7% in the 28S gene and by 16.4% in the *cox*1 gene. This divergence level clearly indicates that our specimens and those sequenced by Hernández-Mena et al. (2017) represent different species. Neither the specimens sequenced by Hernández-Mena et al. (2017), nor our specimens came from the type-host, the brown pelican *Pelecanus occidentalis* Linnaeus. Our material was in

excellent condition and morphologically corresponded very well to the original description. It is unclear whether the identification of specimens used in the two studies as conspecifics stems from the specimen quality or indicates the existence of cryptic species.

Definitive host associations and environmental switches

Based on the broad, essentially cosmopolitan distribution, great diversity of definitive host groups ranging from fishes to mammals, and clear phylogenetic separation from the remaining diplostomoideans, the Cyathocotylidae is undoubtedly a very ancient digenean lineage. Based on the presence of cyathocotylids in crocodilians in Australia, Southeast Asia, Africa and South America, combined with the strong separation of *Suchocyathocotyle* from the remaining members of the family, cyathocotylids likely already existed as a separate lineage in the late Cretaceous (ca. 65-70 mya). The family could be more ancient, but the available data are insufficient for a confident conclusion on this matter.

Our analysis revealed some strongly supported cyathocotylid clades associated with certain groups of definitive hosts. Although cyathocotylids are not particularly diverse in crocodilian hosts, the basal position and strong separation of *Suchocyathocotyle* (Suchocyathocotylinae subfam. n.) from other members of the family likely reflects the ancient nature of their host association rather than a secondary host switching event.

The subfamily Szidatiinae is represented in our tree by *Gogatea* (parasites of snakes) and *Neogogatea* (parasites of birds) and *Holostephanoides* (parasites of fishes) forming a 100% supported clade in BI and 73% in ML. The placement of *Holostephanoides ictaluri*, a parasite of freshwater fishes, in the Szidatiinae represents a significant secondary host switching event, in this case from tetrapods to fish. While this is certainly a very rare event, it has occurred in a

variety of digenean groups. For example, a few microphallid species have transitioned to parasitism in fishes (Gibson, 1996), while members of *Caballerotrema* Prudhoe, 1960 are the only echinostomatoidean digeneans that secondarily switched to parasitism of freshwater fishes (Tkach et al., 2016). Thus, the evolutionary history of the Szidatiinae sub-clade has included at least two major host switching events. Based on the fact that almost all other members of the Cyathocotylinae and Szidatiinae that appeared as sister groups in our analysis (Fig. 1) are parasitic in birds, it is somewhat plausible to hypothesize the general direction of host switching from avian hosts to other vertebrates. However, we abstain here from any definitive conclusions until more taxa of the Szidatiinae can be included in phylogenetic analysis.

Braunininae also form a very strongly supported clade that includes cyathocotylids that transitioned to parasitism of marine mammals, namely dolphins. This shift was accompanied by significant morphological changes; the phylogenetic position and systematic history of *Braunina* was discussed in detail above.

Lastly, the 100% BI and 99% ML supported clade of *Mesostephanus* represents the subfamily Prohemistominae in our analysis. Species included in this study are all parasitic in water birds, but some members of the subfamily are known from other vertebrates such as reptiles. It is difficult to speculate on the exact nature of these associations until a more detailed phylogeny of the Cyathocotylidae becomes available. The systematic position and definitive hosts of Cyathocotylidae sp. (GenBank MH257776) sequenced from a cercaria is presently unknown.

Along with the multiple definitive host switches that occurred in the evolutionary history of the family, cyathocotylids have also transitioned more than once between freshwater and marine environments. While what is currently known about their geographic distribution as well

as definitive and first intermediate hosts strongly supports freshwater life cycles among the ancestral cyathocotylids, members of the former family Brauninidae and some of the prohemistomatine taxa have switched to saltwater life cycles.

Finally, mapping the geographic distribution of the taxa used in our analysis onto the phylogenetic tree did not reveal clades strongly associated with distinct biogeographical realms (Fig. 1). The mosaic nature of the geographic distribution of cyathocotylid taxa across the phylogenetic tree provides support for a likely ancient origin of the group as a whole.

To conclude, this is the first molecular phylogenetic analysis of the Cyathocotylidae that includes a broad variety of taxa from different continents and a wide range of host groups. Importantly, almost all taxa used in our analysis, were represented by adult digeneans, most of them well-fixed and morphologically identifiable. This is the first study to report DNA sequence data for several cyathocotylid taxa and the first to provide molecular data from representatives of the family parasitic in reptiles. Our phylogenetic analysis provided grounds for revisions in the system of the Cyathocotylidae that include transfer of the former Brauninidae into the Cyathocotylidae as a subfamily and erection of the Suchocyathocotylinae subfam. n. Future molecular phylogenetic studies will need to include a higher number of cyathocotylid taxa, including members of the not yet sequenced Muhlinginae and Prosostephaninae, in order to test the monophyly and interrelationships of the currently accepted subfamilies as well as further explore the evolution of their host associations.

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Associated publication

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CHAPTER IV

PHYLOGENY AND SYSTEMATICS OF THE PROTERODIPLOSTOMIDAE REFLECT THE COMPLEX EVOLUTIONARY HISTORY OF THIS ANCIENT DIGENEAN GROUP

Introduction

The Proterodiplostomidae Dubois, 1936 is a relatively small family of diplostomoidean digeneans parasitizing the intestines of reptilian hosts associated with freshwater environments, mostly in the tropical and subtropical regions of the world. The greatest diversity of proterodiplostomids is found in crocodilians, although some parasitize snakes and turtles (Dubois, 1979; Niewiadomska, 2002e). Members of the Proterodiplostomidae are characterized by the presence of a thin- or thick-walled tubule or pouch surrounded by glandular cells associated with the terminal ducts of their reproductive system called a paraprostate (Niewiadomska, 2002e).

Dubois (1936a) established the Proterodiplostomidae for diplostomids from reptiles which possessed a paraprostate. The early systems of the family proposed by Dubois (1936a, 1951) were based on host associations and a wide range of morphological characters including size of the holdfast organ, presence or absence of papillae on the margins of the holdfast organ, distribution of vitelline follicles, and arrangement of terminal reproductive ducts. Dubois (1953) re-visited the systematics of the family and separated the Proterodiplostomidae into two "super-subfamilies" based on host associations (crocodilians and chelonians vs snakes). Byrd & Reiber (1942) and later Brooks et al. (1992) proposed systematic revisions of the Proterodiplostomidae

with a stronger emphasis on the organization of the terminal ducts of the reproductive system. However, Niewiadomska (2002e) in her most recent revision of the Proterodiplostomidae viewed the revision by Brooks et al. (1992) as too preliminary to be broadly adopted as a basis for the current system of the family. According to Niewiadomska (2002e), the Proterodiplostomidae include 17 genera within 5 subfamilies: Massoprostatinae Yamaguti, 1958 (1 genus), Ophiodiplostominae Dubois, 1936 (2 genera), Polycotylinae Monticelli, 1888 (8 genera), Proalarioidinae Sudarikov, 1960 (1 genus) and Proterodiplostominae Dubois, 1936 (5 genera).

Members of the family are distributed on different continents and occur in some of the most ancient groups of amniotic tetrapods, thus representing an extremely interesting model for phylogenetic and phylogeographic studies. However, the current systematics and taxonomy of the Proterodiplostomidae as well as all existing phylogenetic reconstructions of the group (e.g., Brooks, 1979; Brooks & O'Grady, 1989) are morphology-based. The lack of a molecular phylogenetic assessment of the group has prevented us from addressing such intriguing questions as the patterns of their current and past geographic distribution, host associations, or the monophyly of recognized taxa. Likewise, the true interrelationships among the genera within the Proterodiplostomidae remain completely unknown. In fact, the position of the Proterodiplostomidae among other digeneans was tested based only on DNA sequences obtained from metacercariae of only 2 species belonging to 2 of the 17 currently accepted genera, with only weak support (Hernández-Mena et al., 2017; Queiroz et al., 2020). Molecular data are also important as an independent set of characters that may help to assess the relative taxonomic value of morphological characters traditionally used to outline and differentiate among proterodiplostomid taxa including peculiarities of organization of the reproductive system and structure of the holdfast organ.

While significant progress has been recently achieved in the molecular phylogenetics and systematics of the Diplostomoidea Poirier, 1886 and its major constituent lineages (e.g., Blasco-Costa & Locke, 2017; Hernández-Mena et al., 2017; Locke et al., 2018; Achatz et al., 2019b–d; Queiroz et al., 2020), the Proterodiplostomidae remains one of the only diplostomoidean families to receive very little attention in molecular phylogenetic studies. This can be partly explained by the logistic challenges of obtaining fresh material from hosts that are often protected and difficult to collect.

This study is focused on the proterodiplostomids of crocodilians. Based on the available descriptions, taxonomic revisions, and checklists (Dubois, 1979; Catto & Amato, 1994; Tellez, 2014), there are 5 named species of proterodiplostomids belonging to 4 genera reported from crocodilians in the Nearctic: *Archaeodiplostomum acetabulata* (Byrd et Reiber, 1942), *Crocodilicola pseudostoma* Willemoes-Suhm 1870, *Polycotyle ornata* Willemoes-Suhm 1870, *Pseudocrocodilicola americaniense* Byrd et Reiber 1942, and *Pseudocrocodilicola georgiana* Byrd et Reiber 1942. There are 11 species of proterodiplostomids belonging to 7 genera known from crocodilians in the Neotropics: *Cr. pseudostoma*, *Cystodiplostomum hollyi* Dubois, 1936, *Herpetodiplostomum caimancola* (Dollfus, 1935), *Mesodiplostomum gladiolum* Dubois, 1936, *Paradiplostomum abbreviatum* (Brandes, 1888), *Prolecithodiplostomum constrictum* Dubois, 1936, *Proterodiplostomum breve* Catto et Amato, 1994, *Proterodiplostomum globulare* Catto et Amato, 1994, *Proterodiplostomum longum* (Brandes, 1888), *Proterodiplostomum medusae* (Dubois, 1936), *Proterodiplostomum tumidulum* Dubois, 1936, and *Pseudoneodiplostomum groschafti* Moravec, 2001. In the Afrotropics, there are only 2 species of proterodiplostomids belonging to a single genus that parasitize crocodilians: *Pseudoneodiplostomum bifurcatum* (Wedl, 1861) and *Pseudoneodiplostomum thomasi* (Dollfus, 1935). A further 3 species of

proterodiplostomids parasitize crocodilians in the Indomalayan region, each belonging to a separate genus: *Capsulodiplostomum crocodilinum* Dwivedi, 1966, *Herpetodiplostomum gavialis* (Narain, 1930), and *Pseudoneodiplostomum siamense* (Poirier, 1886). No proterodiplostomids have been previously reported from crocodilians in Australia.

In this study, we collected numerous specimens of multiple proterodiplostomid species from 4 species of crocodilian hosts in Australia, Brazil, South Africa, and the U.S.A., in addition to specimens of *Heterodiplostomum lanceolatum* Dubois, 1936 from a frog and a snake from Brazil. We use partial sequences of the nuclear large ribosomal subunit RNA gene (28S) and the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) gene to analyze the phylogenetic position of the Proterodiplostomidae, test its monophyly, and examine the interrelationships among its constituent taxa. In addition, we describe a new genus and two new species of proterodiplostomids from the American alligator *Alligator mississippiensis* (Daudin), re-evaluate some current proterodiplostomid genera, and provide an updated key for the identification of proterodiplostomids to genus level.

Materials & Methods

Several of the genera discussed in the present work have very similar spellings, which prevents the standard use of the first or first and second letters for abbreviation. As such, we use the following abbreviations to refer to genera: *Al.* – *Alligator* Cuvier; *Ar.* – *Archaeodiplostomum* Dubois, 1944; *Co.* – *Crocodylus* Laurenti; *Cr.* – *Crocodilicola* Poche, 1926; *Cy.* – *Cystodiplostomum* Dubois, 1936; *He.* – *Heterodiplostomum* Dubois, 1936; *Me.* – *Mesodiplostomum* Dubois, 1936; *Ne.* – *Neocrocodilicola* n. g.; *Pa.* – *Paradiplostomum* La Rue, 1926; *Pe.* – *Pseudoneodiplostomum* Dubois, 1936; *Po* – *Polycotyle* Willemoes-Suhm 1870; *Pp.*

– *Paraproterodiplostomum* n. g.; *Pr.* – *Proterodiplostomum* Dubois, 1936; *Ps.* – *Pseudocrocodilicola* Byrd & Reiber, 1942; *Pt.* – *Proteroduboisia* n. g.; *Pu.* – *Pseudoneodiplostomoides* Yamaguti, 1954.

Specimens

Adult or immature specimens belonging to the Proterodiplostomidae were collected from the intestines of the following hosts: *Al. mississippiensis* from the Pascagoula Wildlife Management Area, Jackson Co., Mississippi, U.S.A. (30°37'07.2"N, 88°37'08.9"W), between 2004 and 2015; yacare caiman *Caiman yacare* Daudin, yellow-bellied liophis snake *Erythrolamprus poecilogyrus* (Wied-Neuwied) and Cei's white lipped frog *Leptodactylus chaquensis* Cei from Fazenda Retiro Novo, Pantanal, Municipality of Nossa Senhora do Livramento, Mato Grosso State, Brazil, in 2016 and 2019; spectacled caiman *Caiman crocodilus* Linnaeus from the vicinity near Iquitos, Peru in 2016 (kindly provided by Dr. Stephen Bullard, Auburn University); Australian freshwater crocodile *Crocodylus johnstoni* Krefft from Daly River near Oolloo Crossing, Northern Territory, Australia (14°00.31′S, 131°14.46′E) in 2006; and Nile crocodile *Crocodylus niloticus* Laurenti from the Olifants River, Limpopo province (24°3'S, 31°13'E) and Crocodile River, Mpumalanga province, South Africa (25°27'S, 31°58'E) in 2010 and Flag Boshielo Dam, Marble Hall, Limpopo province (24°51'00.5"S, 29°22'55.8"E), South Africa in 2016. In addition, proterodiplostomid metacercaria was collected from mesenteries of the Mississippi green water snake *Nerodia cyclopion* (Duméril, Bibron et Duméril) and an immature proterodiplostomid was obtained from the intestine of the banded water snake *Nerodia fasciata* (Linnaeus) from the Pascagoula Wildlife Management Area, Jackson Co., Mississippi, U.S.A. (30°38'16.5"N, 88°36'35.9"W) in 2011–2012 (Table 3). Live

digeneans from *Co. niloticus* were killed with hot saline, fixed in 10% formalin, and transferred to 70% ethanol. Dead digeneans from the frozen carcass of the Nile crocodile from Flag Boshielo Dam were immediately fixed in 80% ethanol.

Historically, the muscular structure surrounding one or more terminal parts of the reproductive system (e.g., the paraprostate, ejaculatory duct, hermaphroditic duct, metraterm or a combination of the above) in some proterodiplostomids was called a secondary muscular pouch, a muscular sac, a muscular bulb or a capsule. These terms were used without a proper definition or distinct separation between them. Since all these terms refer to structures with a somewhat similar organization and topology, differing only in size or their level of development, we use the unified term "muscular pouch" for these structures.

Type and voucher specimens are deposited in the collection of the Harold W. Manter Laboratory (HWML), University of Nebraska State Museum, Lincoln, NE, U.S.A. or the Museu Paraense Emílio Goeldi (MPEG), Belém, Pará State, Brazil (Table 3). For comparative purposes we examined specimens of *Cr. pseudostoma* from *Crocodylus moreletii* Duméril et Bibronin collected in Mexico and deposited by Vernon Thatcher in the HWML (accession number 21420).

Phylogenetic analyses

Sequences were initially aligned using ClustalW implemented in MEGA7 software (Kumar et al., 2016). The position of proterodiplostomid genera among other diplostomoidean families was studied using an alignment that included newly obtained 28S sequences of 12 proterodiplostomid taxa, previously published sequences of *Cr. pseudostoma*, *He. lanceolatum*, 17 representatives of the Diplostomidae Poirier, 1886, and 13 taxa of the Strigeidae Railliet,

1919. *Suchocyathocotyle crocodili* (Yamaguti, 1954) was used as an outgroup based on the phylogeny published by Achatz et al. (2019d).

Interrelationships within the Proterodiplostomidae were studied using a second alignment of 28S sequences along with an alignment of *cox*1 sequences. *Alaria mustelae* Bosma, 1931 was used an outgroup in both alignments based on the previously published phylogenies and the results of our phylogeny based on the first 28S alignment (see above). The second alignment of the Proterodiplostomidae included newly obtained sequences of 19 proterodiplostomid species and previously published sequences of *Cr. pseudostoma* and *He. lanceolatum*. The *cox*1 alignment included newly obtained sequences of 18 proterodiplostomid species and a single previously published sequence of *Cr. pseudostoma*. Despite all our efforts, we were unable to successfully amplify and sequence *cox*1 for *Me. gladiolum* and *Pseudoneodiplostomum gabonicum* Dubois, 1948.

Phylogenetic analyses were conducted using Bayesian inference (BI) as implemented in MrBayes Ver. 3.2.6 software (Ronquist & Huelsenbeck, 2003). The general time-reversible model with estimates of invariant sites and gamma-distributed among-site variation (GTR + I + G) was identified as the best-fitting nucleotide substitution model for all datasets using MEGA7. Bayesian inference analysis for both 28S datasets were performed using MrBayes software as follows: Markov chain Monte Carlo (MCMC) chains were run for 6,000,000 generations with sample frequency set at 1,000. Bayesian inference analysis for the *cox*1 dataset was performed using MrBayes software as follows: Markov chain Monte Carlo (MCMC) chains were run for 3,000,000 generations with sample frequency set at 1,000. Log-likelihood scores were plotted and only the final 75% of trees were used to produce the consensus trees. The number of generations for each analysis was considered sufficient as the standard deviation stabilized below

Table 3. List of proterodiplostomid species sequenced in the present study including their host species, geographical origin of material, morphological voucher numbers and GenBank accession numbers. HWML: Harold W. Manter Laboratory, Lincoln, Nebraska, U.S.A.; MPEG: Museu Paraense Emílio Goeldi, Belém, Brazil. †Previously *Pseudocrocodilicola*. ‡Previously *Proterodiplostomum*

Digenean taxa	Host species	Geographic origin	Life stage	Museum No.	Accession numbers	
					28S	*cox*1
Archaeodiplostomum overstreeti n. sp.	*Alligator mississippiensis*	U.S.A.[1]	Adult	HWML 216298, 216299	MT622323	MT603590
Archaeodiplostomum overstreeti n. sp.	*Nerodia fasciata*	U.S.A.[2]	Metacercaria	–	MT622324	MT603591
Archaeodiplostomum overstreeti n. sp.	*Nerodia cyclopion*	U.S.A.[2]	Metacercaria	–	MT622325	MT603592
Cystodiplostomum hollyi	*Caiman yacare*	Brazil[3]	Adult	HWML 216300; MPEG 00251–00253	MT622326–MT622329	MT603593–MT603595
Cystodiplostomum sp.	*Caiman yacare*	Brazil[3]	Adult	–	MT622330	MT603596
Heterodiplostomum lanceolatum	*Leptodactylus chaquensis*	Brazil[3]	Metacercaria	–	MT622331	MT603597
Heterodiplostomum lanceolatum	*Erythrolamprus poecilogyrus*	Brazil[3]	Adult	HWML 216301	–	MT603598
Mesodiplostomum gladiolum	*Caiman yacare*	Brazil[3]	Adult	HWML 216302	MT622332	–
Neocrocodilicola georgiana†	*Alligator mississippiensis*	U.S.A.[1]	Adult	HWML 216303, 216304	MT622333–MT622336	MT603599–MT603602
Paradiplostomum abbreviatum	*Caiman yacare*	Brazil[3]	Adult	HWML 216305; MPEG 00254, 00255	MT622337	MT603603
Polycotyle ornata	*Alligator mississippiensis*	U.S.A.[1]	Adult	HWML 216306–216308	MT622338–MT622340	MT603604–MT603606
Proterodiplostomum longum	*Caiman crocodilus*	Peru[4]	Adult	HWML 216309	MT622341	MT603607
Proterodiplostomum medusae	*Caiman yacare*	Brazil[3]	Adult	HWML 216310, 216311; MPEG 00258–00260	MT622342–MT622344	MT603608–MT603610
Proterodiplostomum sp.	*Caiman yacare*	Brazil[3]	Adult	HWML 216312	MT622345	MT603611
Proteroduboisia globulare‡	*Caiman yacare*	Brazil[3]	Adult	HWML 216313; MPEG 00256, 00257	MT622346–MT622353	MT603612–MT603617

Table 3. Continued.

Digenean taxa	Host species	Geographic origin	Life stage	Museum No.	Accession numbers	
					28S	*cox*1
Pseudocrocodilicola americaniense	*Alligator mississippiensis*	U.S.A.[1]	Adult	HWML 216314	MT622354–MT622356	MT603618–MT603620
Pseudoneodiplostomoides crocodilarum	*Crocodylus johnstoni*	Australia[5]	Adult	HWML 216315	MT622357	MT603621
Pseudoneodiplostomum bifurcatum	*Crocodylus niloticus*	South Africa[6, 7, 8]	Adult	HWML 216316, 216317	MT622358–MT622362	MT603622
Pseudoneodiplostomum gabonicum	*Crocodylus niloticus*	South Africa[6, 8]	Adult	HWML 216318, 216319	MT622363–MT622364	–
Pseudoneodiplostomum cf. *siamense*	*Crocodylus johnstoni*	Australia[5]	Adult	–	MT622365	MT603623
Pseudoneodiplostomum thomasi	*Crocodylus niloticus*	South Africa[6]	Adult	HWML 216320	MT622366–MT622367	MT603624–MT603625
Paraproterodiplostomum currani n. g., n. sp.	*Alligator mississippiensis*	U.S.A.[1]	Adult	HWML 216321–216323	MT622368–MT622369	MT603626

[1] Pascagoula Wildlife Management Area, Jackson Co., Mississippi, U.S.A. (30°37'07.2"N, 88°37'08.9"W)
[2] Pascagoula Wildlife Management Area, Jackson Co., Mississippi, U.S.A. (30°38'16.5"N, 88°36'35.9"W)
[3] Fazenda Retiro Novo, Pantanal, Municipality of Nossa Senhora do Livramento, Mato Grosso State, Brazil (16°21'53"S, 56°17'31"W)
[4] Vicinities near Iquitos, Peru
[5] Daly River near Oolloo Crossing, Northern Territory, Australia (14°00.31'S, 131°14.46'E)
[6] Crocodile River, Mpumalanga province, South Africa (25°27'S, 31°58'E)
[7] Olifants River, Limpopo province, South Africa (24°3'S, 31°13'E)
[8] Flag Boshielo Dam, Marble hall, Limpopo province, South Africa (24°51'00.5"S, 29°22'55.8"E)

0.01 in all analyses. Pairwise sequence comparisons were done for sequences included in both 28S and *cox*1 analyses with assistance of MEGA7 software.

Results

Molecular phylogeny

Upon trimming to the length of the shortest sequence obtained from GenBank, the first 28S alignment, which included proterodiplostomids along with members of other diplostomoidean families, was 1,104 bp long; 19 nucleotide positions were excluded due to ambiguous homology. In the phylogenetic tree resulting from the BI analysis, all members of the Proterodiplostomidae formed a strongly supported (96%) monophyletic clade (Fig. 2). This clade was overall very well resolved with high support for almost all topologies. *He. lanceolatum* formed a sister branch to all other members of the Proterodiplostomidae, although the latter cluster had a somewhat low support (82%). A more detailed analysis of the interrelationships within the Proterodiplostomidae is provided below. Similar to other recent molecular phylogenies of the Diplostomoidea, the currently accepted Diplostomidae and Strigeidae were non-monophyletic. This was demonstrated and discussed in several recent works (e. g., Blasco-Costa & Locke, 2017; Hernández-Mena et al., 2017; Locke et al., 2018; Achatz et al., 2019b–d, 2020b; Queiroz et al., 2020); therefore, we do not describe details here.

The second 28S alignment containing only proterodiplostomids was 1,102 bp long after trimming to the length of the shortest sequence; 15 nucleotide positions were excluded due to ambiguous homology and indels. The phylogenetic tree resulting from the BI analysis of the second 28S alignment was well-resolved, except for a basal polytomy which included 4 strongly supported clades (Fig. 3).The highly supported (99%) clade I contained the majority of

proterodiplostomid taxa and was divided into 2 major sub-clades. The first major sub-clade of clade I included all Nearctic species collected from American alligators in Mississippi (91% support) and species of a clade of *Pseudoneodiplostomoides* and *Pseudoneodiplostomum* from crocodiles in Africa and Australia. Within the clade of proterodiplostomids from alligators, *Paraproterodiplostomum currani* n. g., n. sp. formed a sister branch to a 100% supported clade comprising the remaining taxa (Fig. 3). Among those, *Po. ornata* + *Neocrocodilicola georgiana* n. comb. (previously in *Pseudocrocodilicola*; see discussion below) formed a rather weakly supported clade (85%), whereas *Ps. americaniense* and *Archaeodiplostomum overstreeti* n. sp. formed a clade without meaningful support.

Pseudoneodiplostomoides crocodilarum (Tubangui & Masiluñgan, 1936) collected from Australian freshwater crocodiles formed a sister branch (100% support) to *Pseudoneodiplostomum* spp. in the clade of proterodiplostomids collected from *Co. johnstoni* and *Co. niloticus*, correspondingly. *Pseudoneodiplostomum* cf. *siamense* collected from Australian freshwater crocodiles formed a sister branch (100% support) to a strongly supported clade (100%) including the 3 *Pseudoneodiplostomum* species from Nile crocodiles in South Africa.

The second major sub-clade of clade I (100% support) included members of *Cystodiplostomum* and *Proterodiplostomum* from caimans in the Neotropics. Members of each of the two genera formed corresponding 100% supported clades. Within the *Proterodiplostomum* clade the sequence of an unidentified, immature *Proterodiplostomum* sp. formed a sister branch to the 92% supported clade of *Pr. longum* + *Pr. medusae*.

The 100% supported clade II included *Pa. abbreviatum* that appeared basal to the 100% supported group of *Cr. pseudostoma* + *Me. gladiolum*. Clade III included only *Proteroduboisia*

globulare n. comb. (previously in *Proterodiplostomum*; see discussion below) from a caiman collected in Pantanal, Brazil, while the strongly supported (100%) small clade IV comprised two species of *Heterodiplostomum* from a frog and snake in Brazil (Fig. 3).

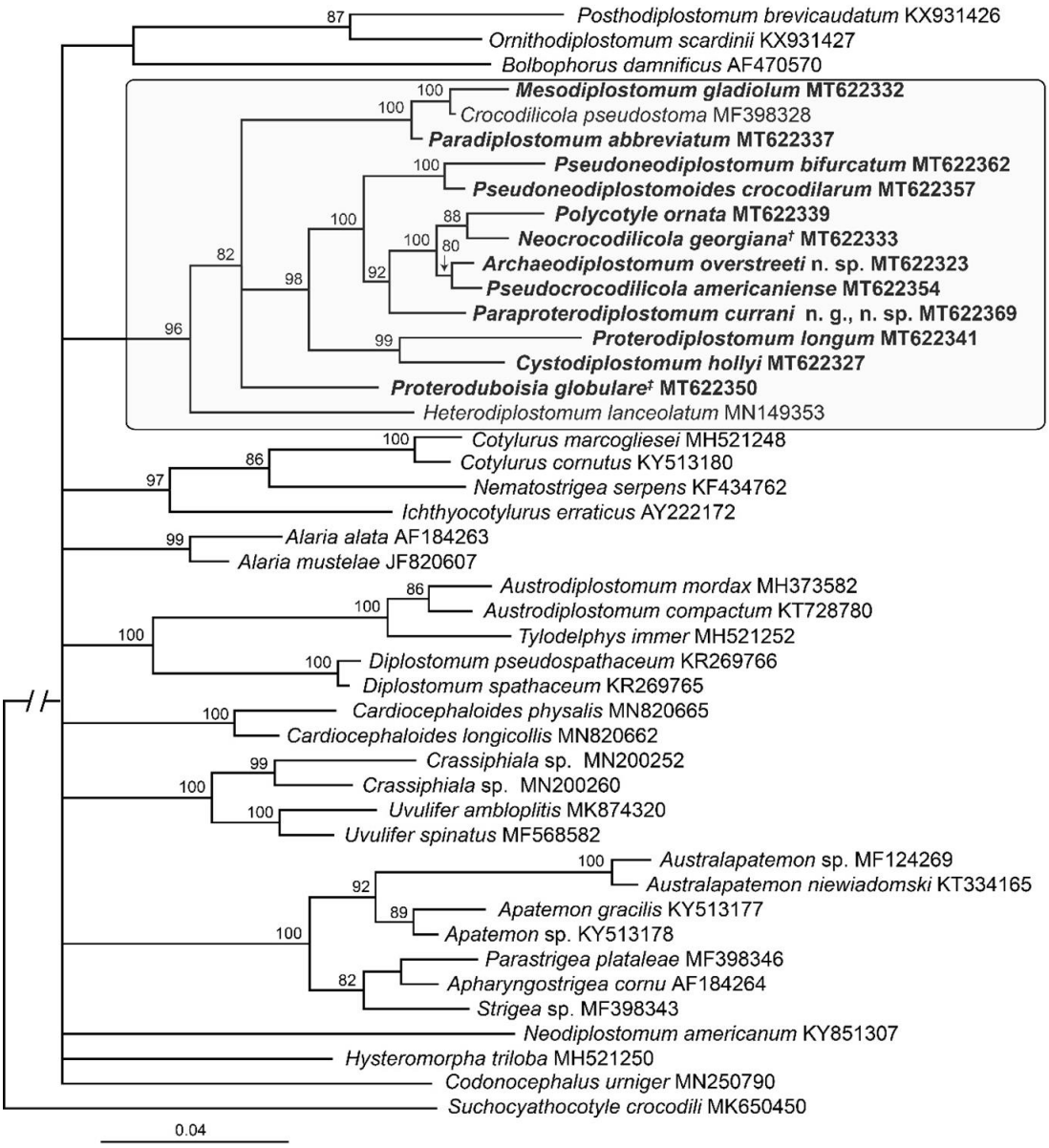


Figure 2. Molecular phylogeny of the Diplostomoidea with a focus on the Proterodiplostomidae resulting from Bayesian inference (BI) analysis based on the partial sequences of the nuclear 28S rRNA gene. Bayesian inference posterior probability values are shown above branches; support values lower than 0.80 (80%) are not shown. GenBank accession numbers are provided after the names of species. The scale bar indicates the number of substitutions per site. Newly generated sequences are highlighted in bold; shaded rectangle indicates the taxa belonging to the Proterodiplostomidae. (after Tkach et al., 2020)

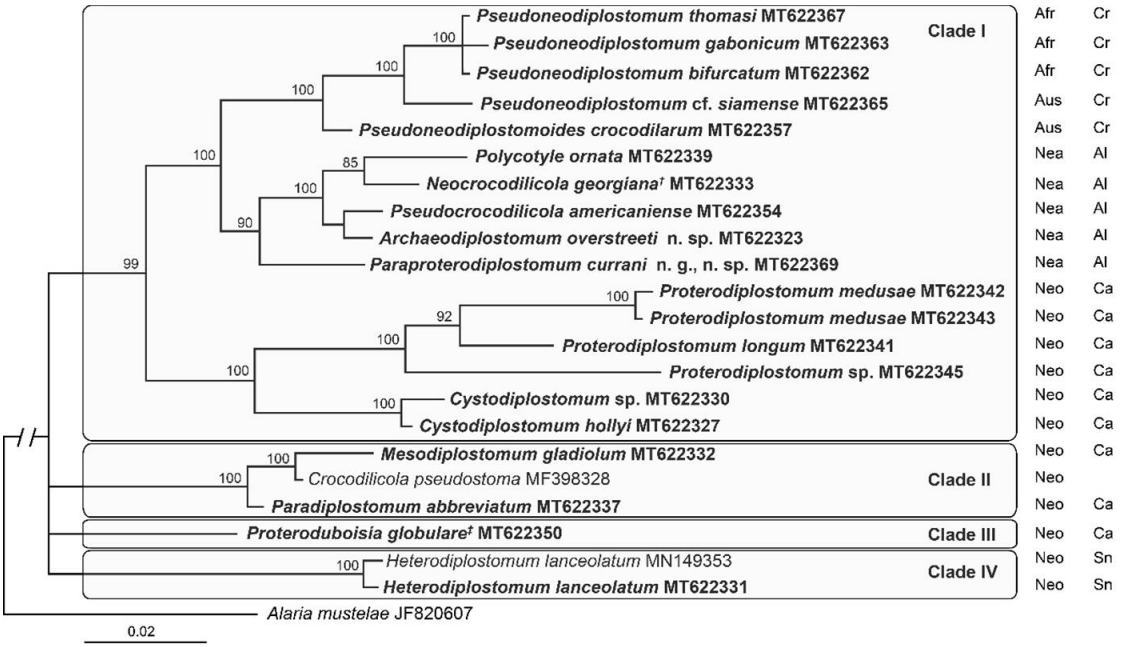


Figure 3. Phylogenetic relationships between the taxa of the Proterodiplostomidae resulting from Bayesian inference (BI) analysis based on the partial sequences of the nuclear 28S rRNA gene. Bayesian inference posterior probability values are shown above branches; support values lower than 0.80 (80%) are not shown. The scale bar indicates the number of substitutions per site. Newly generated sequences are highlighted in bold; rectangles indicate the four major monophyletic clades. GenBank accession numbers are provided after the names of species. Biogeographical realms and definitive host groups are indicated in two columns on the right. Abbreviations for biogeographical realms: Afr, Afrotropical realm; Aus, Australasian realm; Nea, Nearctic realm; Neo, Neotropical realm. Abbreviations for definitive host groups: Cr, true crocodiles (*Crocodylus*), Al, alligators, Ca, caimans, Sn, snakes. (after Tkach et al., 2020)

Upon trimming to the length of the shortest sequence the *cox*1 mtDNA alignment was 520 bp long; no sites were excluded from the analysis. In the phylogenetic tree resulting from the BI analysis (Fig. 4), the topology of the Proterodiplostomidae was much less resolved and differed slightly from the topology in the 28S analyses. Clades II, III, and IV remained the same as in the 28S tree, but clade I split into 6 independent (if low support values are ignored) clades in a polytomy in the *cox*1 tree. The majority, but not all, of the well-supported clades in the *cox*1 tree represented individual proterodiplostomid genera, namely: 1) two *Cystodiplostomum* species (100%); 2) three *Proterodiplostomum* spp. (100%); 3) *Pt. globulare* n. comb.; 4) *He. lanceolatum*; 5) *Pa. abbreviatum* + *Cr. pseudostoma* (100%); 6) *Po. ornata* + *Ne. georgiana* n.

comb. + *Ps. americaniense* + *Ar. overstreeti* n. sp. (100%); 7) *Pseudoneodiplostomum* spp. (93%); 8) *Pp. currani* n. g., n. sp.; 9) *Pu. crocodilarum* (Fig. 4).

It is worth noting that *Ps. americaniense* and *Ne. georgiana* n. comb. (previously in *Pseudocrocodilicola*; see discussion below) formed a 91% supported clade with *Ar. overstreeti* n. sp.; however, the internal topology within this clade was unresolved.

The 3 sequences of *Cy. hollyi* along with the 2 sequences each of *Pt. globulare* n. comb., *He. lanceolatum*, *Ne. georgiana* n. comb. and *Pe. thomasi* formed their own respective 100% supported clades (Fig. 4).

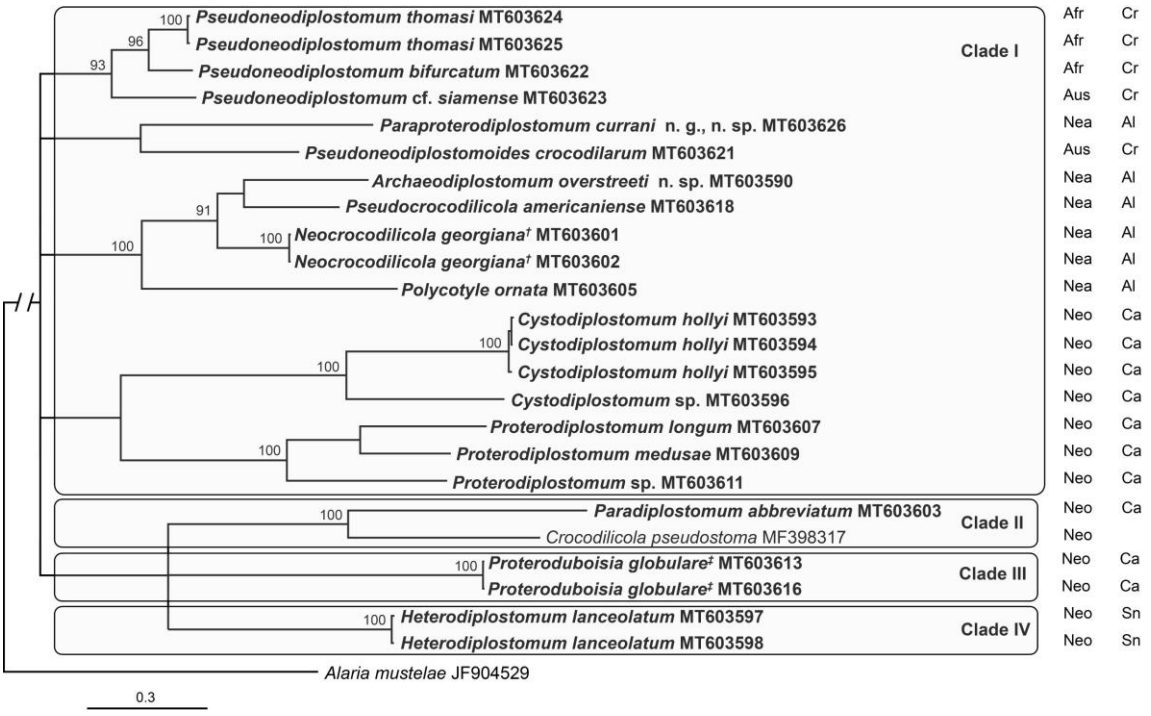


Figure 4. Phylogenetic relationships between the taxa of the Proterodiplostomidae resulting from Bayesian inference (BI) analysis based on the partial sequences of the mitochondrial *cox*1 gene. Bayesian inference posterior probability values are shown above branches; support values lower than 0.80 (80%) are not shown. The scale bar indicates the number of substitutions per site. Newly generated sequences are highlighted in bold; rectangles indicate the taxa belonging to 4 major monophyletic clades in the 28S tree. GenBank accession numbers are provided after the names of species. Biogeographical realms and definitive host groups are indicated in two columns on the right. Abbreviations for biogeographical realms: Afr, Afrotropical realm; Aus, Australasian realm; Nea, Nearctic realm; Neo, Neotropical realm. Abbreviations for definitive host groups: Cr, true crocodiles (*Crocodylus*), Al, alligators, Ca, caimans, Sn, snakes. (after Tkach et al., 2020)

Genetic variation

The pairwise nucleotide comparison of proterodiplostomid sequences of 28S showed an overall low divergence among genera (0.5–6.6% or 5–73 bases out of 1,106). The pairs *Ar. overstreeti* n. sp. + *Ps. americaniense* and *Cr. pseudostoma* + *Pa. abbreviatum* had the lowest intergeneric divergence difference in the 28S sequences (0.5% or 5–6 bases). The greatest intergeneric divergence in the 28S sequences (6.6%) was found in the pairs *Pr. medusae* (GenBank MT622342) + *He. lanceolatum* (GenBank MN149353), *Me. gladiolum* + *Pr. longum* and *Me. gladiolum* + *Pr. medusae* (GenBank MT622342).

The interspecific genetic divergence among congeneric species in the 28S sequences varied greatly across different genera. Our two *Cystodiplostomum* species showed only 0.4% (4 bases) difference in their 28S sequences and *Pseudoneodiplostomum* species demonstrated the lowest interspecific divergence in the 28S sequences among congeners at 0–1% or 0–11 bases. At the same time, members of *Proterodiplostomum* as currently accepted, differed by 2.3–4.3% (25–48 bases) of their 28S sequences.

We did not detect any intraspecific variation in 28S sequences in the majority of species with multiple sequenced specimens, namely *Ar. overstreeti* n. sp. (n=3), *Po. ornata* (n=3), *Pp. currani* n. g., n. sp. (n=2), *Pt. globulare* n. comb. (n=5), *Ps. americaniense* (n=3), *Ne. georgiana* n. comb. (n=4), *Pe. thomasi* (n=2) and *Pe. bifurcatum* (n=5). Only one specimen of *Pr. medusae* (GenBank MT622342) had a single unambiguous base pair difference compared to GenBank MT622343 and MT622344. It is worth noting our new 28S sequence of *He. lanceolatum* (GenBank MT622331) and the previously published sequence of *He. lanceolatum* (GenBank MN149353) differ by 0.2% (2 bases).

In contrast, *cox*1 sequences demonstrated much greater intergeneric variation ranging from 10.4% (54 bases) between *Ps. americaniense* and *Ne. georgiana* n. comb. to 24.8% (129 bases) between *He. lanceolatum* and *Cystodiplostomum* sp. The intrageneric divergence in *cox*1 sequences ranged from 6.7% (35 bases) between *Pe. thomasi* and *Pe. bifurcatum* to 16.5% (86 bases) between *Proterodiplostomum* sp. and *Pr. longum*.

No intraspecific variation was detected among *cox*1 sequences of *Ar. overstreeti* n. sp., *Cr. pseudostoma*, *Po. ornata*, *Pr. medusae*, *Pt. globulare* n. comb. and *Ps. americaniense*. In species that demonstrated intraspecific variation in *cox*1, it was dramatically lower than the lowest levels of interspecific divergence and varied between 0.2% and 0.6% (1–3 bases) in *Cy. hollyi*, *He. lanceolatum* and *Ne. georgiana* n. comb.

Descriptions of new taxa

Results of our molecular phylogenetic analysis and morphological examination of freshly collected high-quality specimens of proterodiplostomids have revealed the presence of two new species and a new genus in our material from American alligators. Their descriptions are provided below.

Paraproterodiplostomum n. g. Tkach, Achatz et Pulis

Diagnosis: Body bipartite; prosoma elliptical; opisthosoma elongate, cylindrical. Oral and ventral suckers well-developed; pseudosuckers absent; holdfast organ large, elliptical, protruding from prosoma. Pharynx moderately developed; caeca extending to near posterior end of opisthosoma. Testes tandem, similar in size, mostly located in last third of opisthosoma. Paraprostate well-developed, claviform; ejaculatory duct joins paraprostate near its distal end to

form common male efferent duct that opens into genital atrium. Ovary pretesticular. Vitellarium extends from approximately level of ventral sucker to past posterior testis. Metraterm opens separately from common male efferent duct into genital atrium. Genital atrium opening subterminal on dorsal side. Excretory pore terminal. Nearctic. In *Alligator mississippiensis.*

Type- and only species: Paraproterodiplostomum currani n. g., n. sp.

Etymology: The name of the new genus reflects its morphological similarity to *Proterodiplostomum*.

Paraproterodiplostomum currani n. sp. Tkach, Achatz et Pulis

(Fig. 5)

Description [Based on 9 adult specimens; measurements of holotype given in text; measurements of entire series given in Table 4]: Body 6,208 long, consisting of distinct prosoma and opisthosoma; prosoma elliptical, 2,031 long, with maximum width at level of holdfast organ, 965; opisthosoma elongate, cylindrical, 4,177 × 421 wide. Prosoma:opisthosoma length ratio 0.49. Minuscule scale-like tegumental spines covering anterior part of prosoma almost to level of anterior margin of holdfast organ. Oral sucker subterminal, 111 × 119. Pseudosuckers absent. Ventral sucker slightly larger than oral sucker, 131 × 139, located near mid-length of prosoma; oral:ventral sucker width ratio 1:1.17. Holdfast organ posterior to ventral sucker, protruding from prosoma; subspherical or oval with ventral muscular portion, highly variable in shape, occupying almost entire width of prosoma, 941 × 961. Holdfast organ equal to 46% of prosoma length. Proteolytic gland extensive, located at base of holdfast organ. Prepharynx not observed. Pharynx oval, 100 × 80. Osophagus slightly longer than pharynx. Caecal bifurcation in anterior third of prosoma. Caeca slender, extending to near posterior end of opisthosoma.

Testes tandem, entire, mostly located in posterior third of opisthosoma; anterior testis 395 × 324, posterior testis 459 × 312. Seminal vesicle post-testicular, compact, coiled, ventral to posterior testis, continuing as ejaculatory duct before connecting to base of paraprostate to form common male efferent duct. Paraprostate well-developed, claviform, 415 × 128, with proximal end reaching close to posterior testis, surrounded by gland cells. Common male efferent duct opening into genital atrium separately from female opening.

Ovary pretesticular, oval or subspherical 287 × 228. Oötype, Mehlis' gland and uterine seminal receptacle inter-testicular. Vitelline follicles located around holdfast organ in prosoma and extending posteriorly to about level of paraprostate, ventral and lateral to gonads. Vitelline reservoir intertesticular. Uterus ventral to gonads, extending anteriorly from ovary to near junction of prosoma and opisthosoma before turning and extending posteriorly. Metraterm opening into genital atrium separately from common male efferent duct; genital atrium opening subterminal on dorsal side. Uterus contains numerous eggs (85–96 x 46–59). Genital atrium subterminal, on dorsal side. Excretory vesicle not well-observed. Excretory pore terminal.

Taxonomic summary

Type-host: *Alligator mississippiensis* (Daudin) (Crocodilia: Alligatoridae).

Site in host: Small intestine.

Type-locality: Pascagoula Wildlife Management Area, Jackson Co., Mississippi, U.S.A. (30°37'07.2"N 88°37'08.9"W).

Type-material: The type series consists of 9 fully mature specimens deposited in the HWML. Holotype: HWML 216321, labeled ex. *Alligator mississippiensis*, small intestine, Pascagoula

wildlife management area, Jackson Co., Mississippi, U.S.A., 10 July 2015, coll. V. Tkach. Paratypes: HWML 216322, 216323 (lot of 8 slides), labels identical to the holotype.

Etymology: The species is named after Dr. Stephen Curran in recognition of his contributions to trematodology, particularly to our knowledge of the trematodes in the Gulf of Mexico and the Gulf Coast, and his invaluable help and camaraderie in numerous collecting trips in the region and beyond.

Remarks

The new genus can be differentiated from all other known proterodiplostomid genera based on a range of morphological characters. *Paraproterodiplostomum currani* n. g., n. sp. differs from *Heterodiplostomum* by the lack of a muscular pouch surrounding the paraprostate (Figs 5, 6G, S); additionally, *Pp. currani* n. g., n. sp. differs from *He. lanceolatum* by 4.2% (36 bases) in the 28S sequence nucleotide positions and up to 20.8% (107 bases) in *cox*1 sequences. The new genus can be readily differentiated from *Capsulodiplostomum* Dwivedi, 1966 due to the lack of the muscular pouch enclosing the paraprostate, ejaculatory duct and metraterm. Unlike the members of *Mesodiplostomum* and *Proalarioides* Yamaguti, 1933 which lack a visible paraprostate, the new genus has a

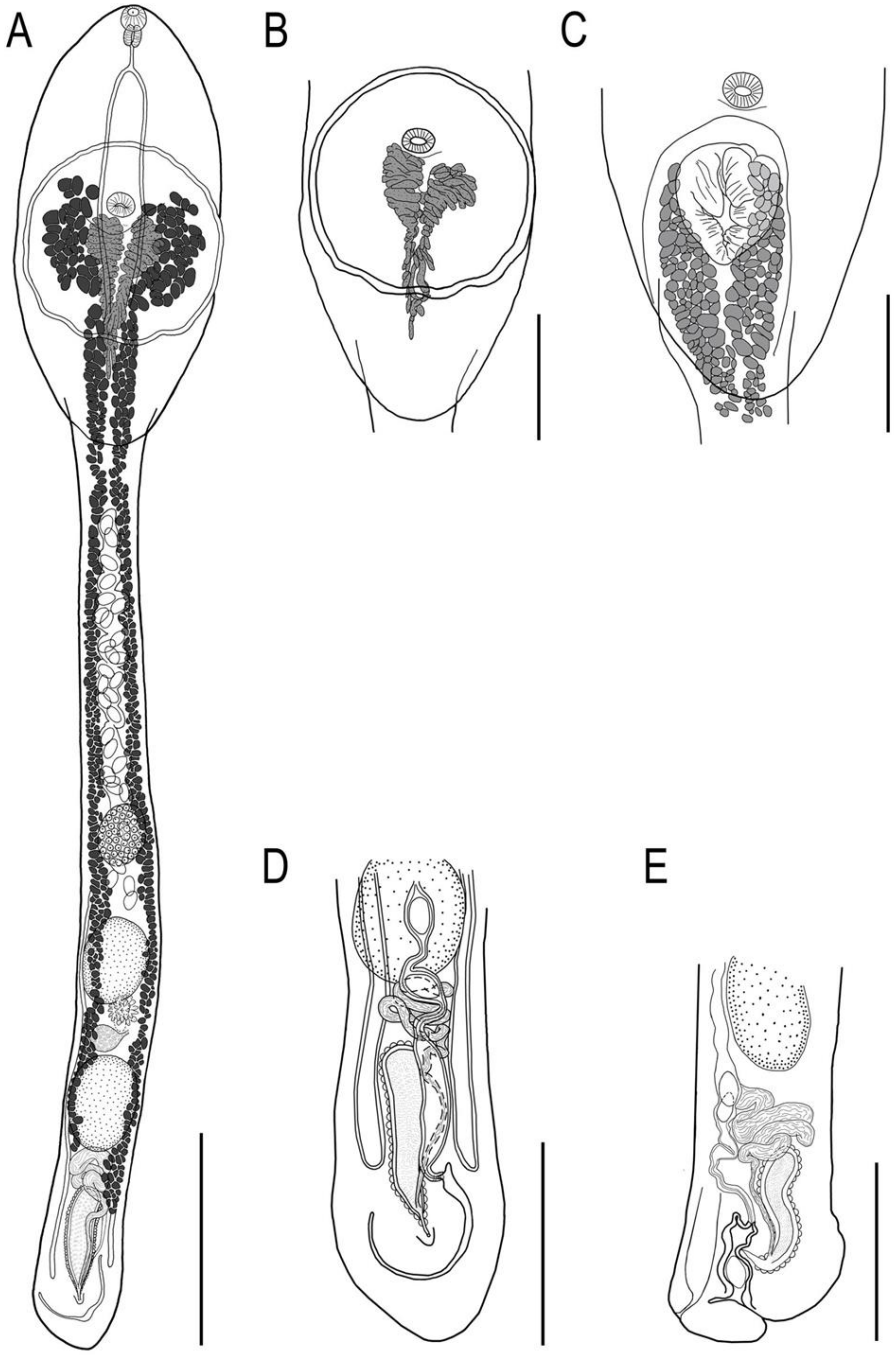
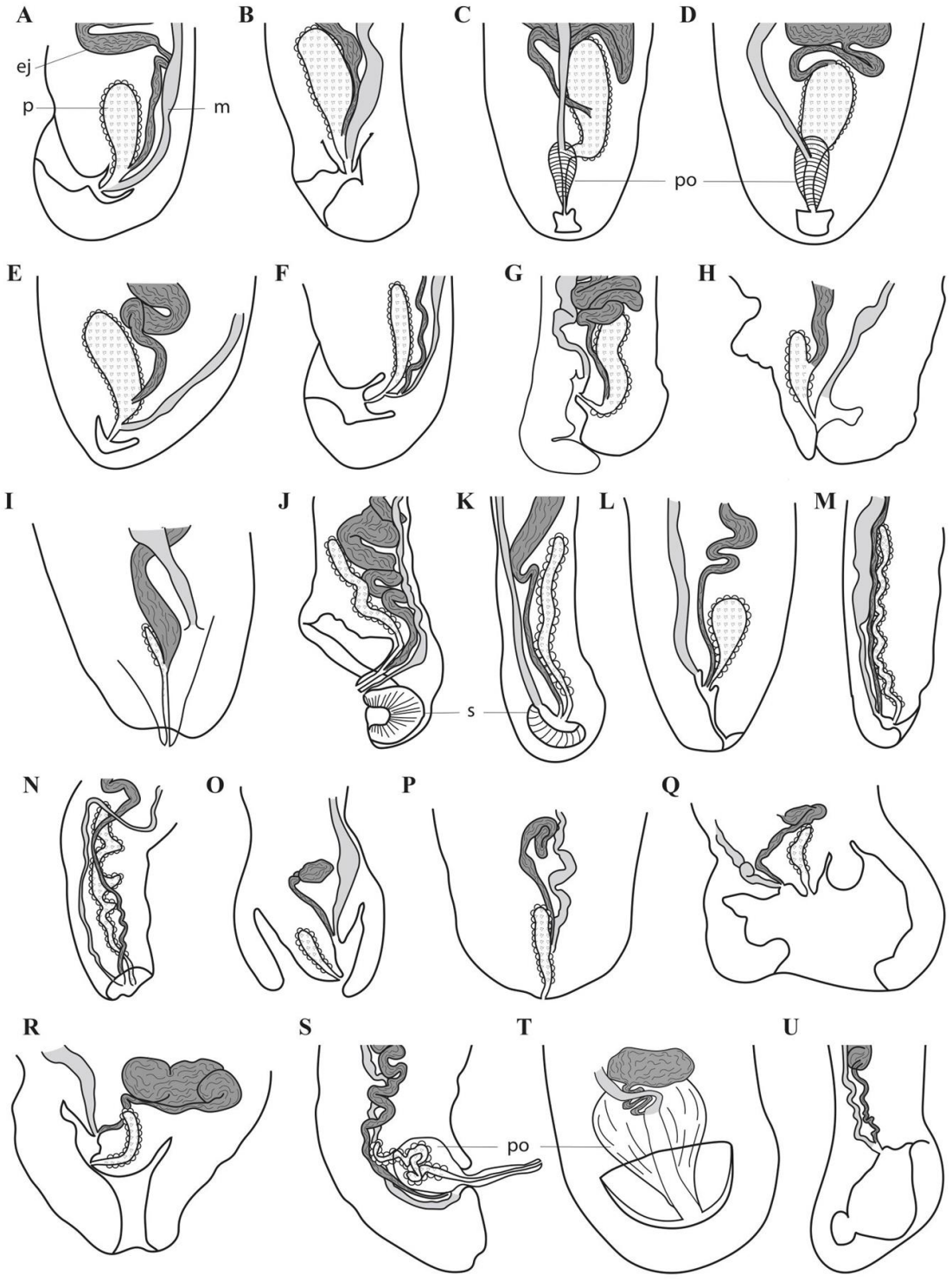


Figure 5. *Paraproterodiplostomum currani* n. sp. **(A)** Ventral view of the holotype; **(B)** Proteolytic gland in the holotype; **(C)** Proteolytic gland in a paratype; **(D)** Posterior end of a paratype showing terminal ducts of the reproductive system, ventral view; **(E)** posterior end of a paratype showing terminal ducts of the reproductive system, lateral view. Scale-bars: A, 1 mm; B, C, D, E, 500 µm. (after Tkach et al., 2020)

well-developed paraprostate (Figs 5, 6G, T, U). The new genus and *Me. gladiolum* differ by 3.8% (42 bases) in the 28S sequences. *Paraproterodiplostomum currani* n. g., n. sp. is easily distinguishable from *Ophiodiplostomum* Dubois, 1936 species based on the relative size of the holdfast organ. In *Pp. currani* n. g., n. sp. the holdfast organ occupies on average 34% (24–46%) of the prosoma, while the holdfast organ of *Ophiodiplostomum* species is relatively larger and occupies approximately half of the prosoma length.

The terminal ducts of the male and female reproductive systems in *Pp. currani* n. g., n. sp. open separately into the genital atrium. In contrast, the metraterm of *Archaeodiplostomum*, *Crocodilicola*, *Polycotyle*, and *Pseudocrocodilicola* species joins the common male efferent duct prior to reaching the genital atrium (Fig. 6A–C, E–G). Whereas the ejaculatory duct in the new genus joins the paraprostate, in *Cheloniodiplostomum* Sudarikov, 1960, *Cystodiplostomum*, *Herpetodiplostomum* Dubois, 1936, *Massoprostatum* Caballero, 1948, *Paradiplostomum*, and *Prolecithodiplostomum*

Figure 6. Topologies of terminal reproductive ducts of representative species of all currently accepted proterodiplostomid genera with the exception of *Capsulodiplostomum* and *Cystodiplostomum*. *Capsulodiplostomum* was omitted due to a lack of any previously published, quality illustrations of the terminal ducts. *Cystodiplostomum* was not drawn separately as the topology of its terminal reproductive ducts is identical to *Prolecithodiplostomum constrictum*. **(A)** *Archaeodiplostomum acetabulatum*, lateral view; **(B)** *Archaeodiplostomum overstreeti* n. sp., lateral view; **(C)** *Pseudocrocodilicola americaniense*, ventral view; **(D)** *Neocrocodilicola georgiana* n. comb., ventral view; **(E)** *Crocodilicola pseudostoma*, ventral view; **(F)** *Polycotyle ornata*, lateral view; **(G)** *Paraproterodiplostomum currani* n. g., .n. sp., lateral view; **(H)** *Pseudoneodiplostomum gabonicum*, lateral view; **(I)** *Pseudoneodiplostomoides crocodilarum*, dorsal view; **(J)** *Proterodiplostomum longum*, lateral view; **(K)** *Proterodiplostomum medusae*, lateral view; **(L)** *Proteroduboisia globulare* n. comb., lateral view; **(M)** *Prolecithodiplostomum constrictum*, lateral view; **(N)** *Massoprostatum longum*, ventral view; **(O)** *Paradiplostomum abbreviatum*, lateral view; **(P)** *Ophiodiplostomum spectabile*, dorsal view; **(Q)** *Cheloniodiplostomum testudinis*, lateral view; **(R)** *Herpetodiplostomum caimancola*, lateral view; **(S)** *Heterodiplostomum lanceolatum*, lateral view; **(T)** *Proalarioides serpentis*, ventral view; **(U)** *Mesodiplostomum gladiolum*, lateral view. A, C–F, after Byrd & Reiber (1942); H, after Dubois (1948); I, after Yamaguti (1954); J, M, P–S, U, after Dubois (1936a); K, L, O, after Catto & Amato (1994); N, T, after Sudarikov (1960b). Abbreviations: b, muscular bulb; ej, ejaculatory duct; m, metraterm; p, paraprostate; s, muscular sucker-like structure. (after Tkach et al., 2020)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Dubois, 1936 the paraprostate opens separately from the ejaculatory duct and metraterm (Figs 5, 6G, M–O, Q, R). The new genus differs from *Archaeodiplostomum*, *Crocodilicola*, *Polycotyle*, *Pseudocrocodilicola*, *Cystodiplostomum*, and *Paradiplostomum* spp. by 1.5–3.9% (16–43 bases) in the 28S sequences and 17.3–22.3 % (90–116 bases) in the *cox*1 sequences.

Paraproterodiplostomum currani n. g., n. sp. clearly differs from *Proterodiplostomum* species by the absence of the sucker-like structure in the genital atrium. Furthermore, the ejaculatory duct of *Paraproterodiplostomum* n. g. joins the paraprostate at its base, whereas the ejaculatory duct of *Proterodiplostomum* does not join the paraprostate. However, the ejaculatory duct of *Proterodiplostomum* may later join the efferent duct of the paraprostate (Figs 5, 6G, J, K). In addition, the sequences of the new genus demonstrate significant differences from *Proterodiplostomum* species in both the 28S (4.8–5.4% or 53–60 bases) and *cox*1 (19–21.3% or 99–116 bases) genes.

The new genus has a well-developed paraprostate compared to the relatively small and weaker developed paraprostate in *Pseudoneodiplostomum.* The two genera can be further differentiated based on the position of the ejaculatory duct and paraprostate juncture. In *Pp. currani* n. g., n. sp. the ejaculatory duct joins the paraprostate at its base, whereas in members of *Pseudoneodiplostomum* the ejaculatory duct joins the paraprostate between its midlength and proximal (anterior) end (Figs 4, 6G, H). In addition, the new genus differs from members of *Pseudoneodiplostomum* by 2.6–2.7% (29–30 bases) in the 28S sequences and by 16.3–18.1% (85–94 bases) in the *cox*1 sequences.

Table 4. Metric characters of *Paraproterodiplostomum currani* (n=9) n. g., n. sp. from Mississippi. Abbreviations: StDev, standard deviation; CV, coefficient of variation.

	Mean	Range	StDev	CV
Overall body length	5779	5210–6466	429.7	7.4
Prosoma length	2139	1947–2362	148.4	6.9
Prosoma width	924	833–1108	97.1	10.5
Opisthosoma length	3736	3368–4198	327.7	8.8
Opisthosoma width	401	354–451	36.7	9.2
Prosoma:opisthosoma length ratio	0.58	0.49–0.65	0.1	10
Forebody length	1015	857–1308	135.2	13.3
Ventral sucker length	116	99–132	13.5	11.6
Ventral sucker width	136	120–164	16.7	12.2
Oral sucker:ventral sucker width ratio	0.83	0.66–0.96	0.11	13.4
Holdfast organ length	716	540–941	157.7	22
Holdfast organ width	695	533–961	180.4	25.9
Anterior margin of holdfast positioned at (% of prosoma length)	0.45	0.31–0.56	0.11	25
Distance between ventral sucker & holdfast organ:prosoma length	0.04	0–0.12	0.05	128.3
Pharynx length	105	98–118	7.1	6.7
Pharynx width	83	78–89	4	4.8
Esophagus length	114	67–162	31	27.2
Anterior testis length	304	221–395	50.1	16.5
Anterior testis width	252	218–324	35	13.8
Posterior testis length	350	287–459	51.4	14.7
Posterior testis width	259	221–312	32.3	12.5
Distance between posterior margin of posterior testis & end of body:opisthosoma length	0.23	0.2–0.27	0.03	11.3
Seminal vesicle length	880	776–978	83	9.4
Paraprostate length	398	344–479	40.5	10.2
Paraprostate width	125	102–143	14.7	11.7
Ovary length	272	215–324	32.2	11.8
Ovary width	194	162–228	19.4	10
Metraterm length	262	228–296	48.1	18.4
Egg number	22	1–36	13.1	63.7
Egg length	92	85–96	2.8	3.1
Egg width	55	46–59	3.9	7.1
Anterior vitellarium-free zone:prosoma length	0.54	0.41–0.77	0.13	24.8
Posterior vitellarium-free zone:opisthosoma length	0.22	0.15–0.29	0.05	22.9

Archaeodiplostomum overstreeti n. sp. Tkach, Achatz et Pulis

(Fig. 7)

Description [Based on 4 adult specimens; measurements of holotype given in text; measurements of entire series given in Table 5]: Body 6,109 long, consisting of prosoma and opisthosoma; prosoma elongate, 3,194 long, much wider than opisthosoma, with maximum width at level of holdfast organ, 959; opisthosoma elongate, cylindrical, 2,915 × 340, similar in length to prosoma; prosoma:opisthosoma length ratio 1.1. Minuscule scale-like tegumental spines covering anterior part of prosoma and reaching level of posterior margin of ventral sucker. Oral sucker subterminal, 138 × 142. Pseudosuckers absent. Prepharynx not observed. Pharynx oval, 79 × 93. Oesophagus approximately twice as long as pharynx. Caecal bifurcation in anterior third of prosoma. Caeca slender, blind, extending to near posterior end of opisthosoma. Ventral sucker 508 × 488, much larger than oral sucker, typically located somewhat anterior to mid-length of prosoma. Oral sucker:ventral sucker width ratio 1:3.4. Holdfast organ 496 × 387, posterior to ventral sucker, located in last third of prosoma, oval with ventral muscular portion. Holdfast organ equal to 16% of prosoma length. Proteolytic gland at base of holdfast organ.

Testes tandem, smooth, mostly located in middle third of opisthosoma; anterior testis 176 × 225, posterior testis 193 × 222. Seminal vesicle post-testicular, elongated, sinuous, continuing as sinuous ejaculatory duct prior to joining base of paraprostate to form common male efferent duct. Paraprostate well-developed, claviform, 220 × 101, surrounded by gland cells. Common male efferent duct and metraterm join to form a common duct almost immediately prior to opening into genital atrium.

Ovary immediately pretesticular, subspherical, 99 × 96. Oötype and Mehlis' gland intertesticular. Seminal receptacle subspherical, immediately dorsal to oötype, smaller than

ovary. Vitelline follicles distributed from level immediately posterior to ventral sucker to immediately anterior to paraprostate, ventral and lateral to gonads. Vitelline reservoir intertesticular. Uterus ventral to gonads, extending anteriorly from ovary to about level of prosoma and opisthosoma before turning and extending posteriorly and eventually transitioning into metraterm. Uterus contains numerous eggs (78–97 × 47–55). Genital atrium subterminal, on dorsal side. Excretory vesicle not observed. Excretory pore terminal.

Taxonomic summary

Type-host: *Alligator mississippiensis* (Daudin) (Crocodilia: Alligatoridae).

Site in host: Small intestine.

Type-locality: Pascagoula Wildlife Management Area, Jackson Co., Mississippi, U.S.A. (30°37'07.2"N, 88°37'08.9"W).

Type-material: The type series consists of 4 fully mature specimens deposited in the HWML. Holotype: HWML 216298, labeled ex. *A. mississippiensis*, small intestine, Pascagoula wildlife management area, Jackson Co., Mississippi, U.S.A., 30°37'07.2"N 88°37'08.9"W, 17 Aug 2010, coll. V. Tkach. Paratypes: HWML 216299 (lot of 3 slides), labels identical to the holotype.

Etymology: The species is named after Dr. Robin Overstreet in recognition of his numerous contributions to helminthology including helminths of crocodilians, and his invaluable help with collection of specimens in Mississippi.

Remarks

The new species clearly belongs to *Archaeodiplostomum* based on the large ventral sucker, a well-developed claviform paraprostate, and an ejaculatory duct that joins the base of paraprostate

to form a common male efferent duct that subsequently merges with the metraterm to form a common duct. At present, *Archaeodiplostomum* includes a single species *Ar. acetabulata.*

Archaeodiplostomum overstreeti n. sp. differs from *Ar. acetabulata* by having a more elongated prosoma compared to the pyriform-shaped prosoma in *Ar. acetabulata* (Fig. 7; Byrd & Reiber, 1942). The new species can also be differentiated from *Ar. acetabulata* by having a longer body (6,109–7,706 in the new species vs 4,800–5,960 in *Ar. acetabulata*) and a typically smaller holdfast organ (493–598 × 387–465 in the new species vs 570–840 × 500–740 in *Ar. acetabulata*). In addition, *Ar. overstreeti* n. sp. has substantially smaller paraprostate (218–255 × 101–112 in the new species vs 310–450 × 120–160 in *Ar. acetabulata*), ovary and testes. Metacercaria of *Ar. overstreeti* n. sp. were recovered and sequenced from *N. fasciata* and *N. cyclopion* in Mississippi. These snakes are common in the areas where the alligators were captured. The specimen sequenced from the *N. fasciata* was found excysted in the stomach, but was not sexually mature. Gut contents of the snake contained unidentifiable fish remnants. The specimen sequenced from the *N. cyclopion* was found in the mesenteries, thus providing the first evidence that snakes can likely act as paratenic hosts for these digeneans, which are parasitic as adults in alligators.

Discussion

Abandonment of the subfamily based system of the Proterodiplostomidae

Our molecular phylogenetic analysis of a broad diversity of proterodiplostomids from a variety of hosts from 4 continents strongly support the monophyly of the Proterodiplostomidae. At the same time, our results do not support the most recent (or any of the previous) systematic arrangement of some of the taxa, particularly the current subfamily structure within the family as

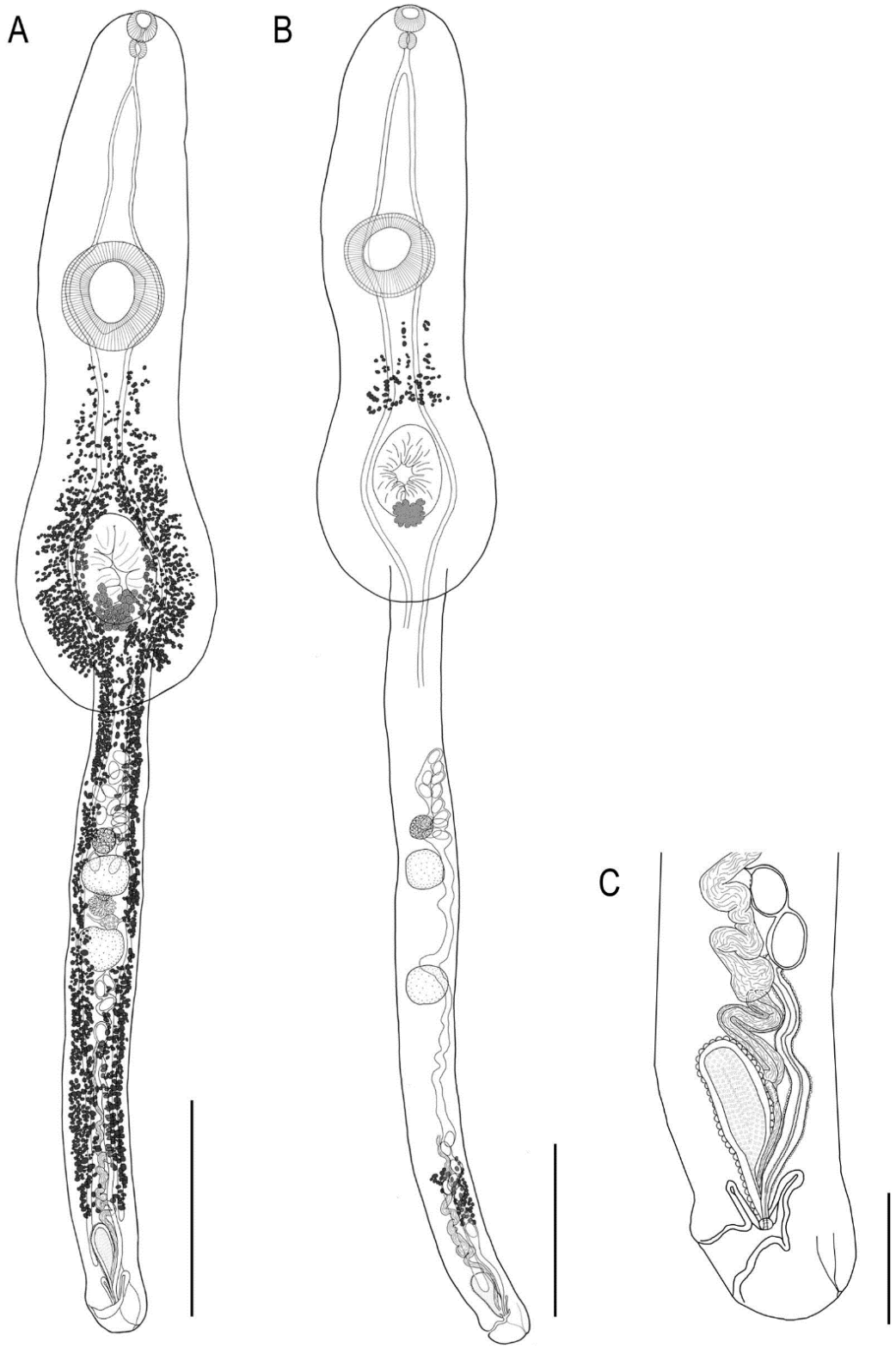

Figure 7. *Archaeodiplostomum overstreeti* n. sp. **(A)** Ventral view of the holotype; **(B)** Ventral view of a paratype, anteriormost and posteriormost vitelline follicles are shown for clarity; **(C)** Posterior end of a paratype showing terminal ducts of the reproductive system, lateral view. Scale-bars: A, B, 1 mm; C, 200 µm. (after Tkach et al., 2020)

Table 5. Metric characters of *Archaeodiplostomum overstreeti* (n=4) n. sp. from Mississippi. Abbreviations: StDev, standard deviation; CV, coefficient of variation.

	Mean	**Range**	**StDev**	**CV**
Overall body length	6753	6109–7706	776.4	11.5
Prosoma length	3260	3063–3418	162.6	5
Prosoma width	929	905–959	27.6	3
Opisthosoma length	3561	2915–4288	581.3	16.3
Opisthosoma width	349	318–390	36.9	10.6
Prosoma:opisthosoma length ratio	0.93	0.8–1.1	0.12	13.3
Forebody length	1371	1293–1430	63.8	4.7
Forebody:body length ratio	0.2	0.18–0.22	0.02	7.55
Oral sucker length	145	128–163	15.1	10.5
Oral sucker width	149	142–156	7	4.7
Ventral sucker length	442	409–508	46.4	10.5
Ventral sucker width	444	412–488	39.2	8.8
Oral sucker:ventral sucker width ratio	0.34	0.29–0.36	0.04	12
Holdfast organ length	522	493–598	50.6	9.7
Holdfast organ width	416	387–465	42.7	10.3
Anterior margin of holdfast positioned at (% of prosoma length)	0.7	0.65–0.74	0.04	10.3
Distance between ventral sucker & holdfast organ:prosoma length	0.28	0.23–0.31	0.04	13.8
Pharynx length	94	79–106	13.7	14.6
Pharynx width	88	85–93	4.2	4.7
Esophagus length	132	107–163	28.4	21.4
Anterior testis length	216	176–243	28.3	13.1
Anterior testis width	220	199–236	19	8.6
Posterior testis length	227	193–248	29.5	13
Posterior testis width	224	199–250	25.5	11.4
Distance between posterior margin of posterior testis & end of body:opisthosoma length	0.52	0.46–0.57	0.05	9
Seminal vesicle length	1279	822–1874	471.5	36.9
Paraprostate length	235	218–255	18.6	7.9
Paraprostate width	109	101–112	6.4	5.9
Ovary length	114	99–129	15.4	13.6
Ovary width	112	96–129	13.5	12.1
Metraterm length	321	285–352	33.9	10.5
Egg number	60	29–84	23	38.3
Egg length	89	78–97	5.8	6.5
Egg width	60	47–55	3.3	6.4

Table 5. Continued.

	Mean	Range	StDev	CV
Anterior vitellarium-free zone:prosoma length	0.52	0.48–0.56	0.04	7
Posterior vitellarium-free zone:opisthosoma length	0.17	0.15–0.19	0.02	10.8

presented by Niewiadomska (2002e). Although, this is true for both 28S and *cox*1-based phylogenies, we primarily rely on the 28S data in our subsequent considerations due to the much higher resolution at the suprageneric level provided by this gene. As has been previously suggested, the analyses based on *cox*1 data produces low resolution and numerous polytomies that most likely resulting from the mutation saturation effect. Considering that the Proterodiplostomidae clearly is an ancient group of digeneans, combined with the fact that crocodilians live in warm climates where parasite life cycles continue throughout the year, these parasites evolved over a great span of evolutionary time in terms of the number of generations. This most likely resulted in a greater mutation accumulation in fast mutating mitochondrial genes leading to lower resolution in the *cox*1 trees compared to those produced by the analyses of the slower mutating 28S gene. As noted by previous authors (e.g., Locke et al., 2018; Queiroz et al., 2020) the usefulness of commonly sequenced nuclear ribosomal and mitochondrial genes for phylogenetic inference at different taxonomic levels varies and necessitates careful assessment.

While the early systematics of the Proterodiplostomidae were based on a variety of characters traditionally used in digenean taxonomy, Brooks et al. (1992) proposed a revised system of the family with an emphasis on the structure of the terminal parts of the reproductive system. These authors split the Proterodiplostomidae based on the following 4 conditions: 1) paraprostate fused with ejaculatory duct and metraterm (referred to as uterus by Brooks et al.

[1992]) opening separately; 2) paraprostate and ejaculatory duct fused, then metraterm fused with common male efferent duct; 3) paraprostate fused first with metraterm and then with ejaculatory duct; 4) paraprostate opening separately. This led Brooks et al. (1992) to propose two subfamilies, the Heterodiplostominae Dubois, 1936 incertae sedis, sedis mutabilis (*Heterodiplostomum* and *Ophiodiplostomum*) and the Proterodiplostominae with the latter divided into 3 tribes: 1) Pseudoneodiplostomini Dubois, 1936 sedis mutabilis (*Neelydiplostomum* Gupta, 1958, currently considered a synonym of *Herpetodiplostomum*, and *Pseudoneodiplostomum*), 2) Pseudocrocodilicolini Byrd & Reiber, 1942 sedis mutabilis (*Archaeodiplostomum, Crocodilicola, Pseudocrocodilicola* and *Polycotyle*), and 3) Proterodiplostomini Dubois, 1936 sedis mutabilis (*Cystodiplostomum, Herpetodiplostomum, Massoprostatum, Mesodiplostomum, Paradiplostomum, Prolecithodiplostomum* and *Proterodiplostomum*).

Our analyses support neither Brooks et al.'s (1992) subfamilies nor the tribes Pseudocrocodilicolini (with a caveat that the *Crocodilicola* sequence in GenBank originated from a metacercaria) and Proterodiplostomini.

In the most recent revision of the Proterodiplostomidae, Niewiadomska (2002e) did not accept the system proposed by Brooks et al. (1992). Her system included 5 subfamilies: Massoprostatinae, Ophiodiplostominae, Polycotylinae, Proalarioidinae, and Proterodiplostominae. Our molecular phylogenetic analyses included 5 genera from the Polycotylinae (*Crocodilicola, Cystodiplostomum, Paradiplostomum*, *Pseudocrocodilicola*, and *Polycotyle*), 4 genera from the Proterodiplostominae (*Archaeodiplostomum, Mesodiplostomum, Pseudoneodiplostomum*, and *Proterodiplostomum*) and 1 genus of the Ophiodiplostominae

(*Heterodiplostomum*). Our analyses revealed the Polycotylinae and Proterodiplostominae to be clearly paraphyletic (Figs 2–4).

Our molecular phylogenetic results do not support any of the previously proposed systems of the Proterodiplostomidae including the most recent system proposed by Niewiadomska (2002e). The use of the organization of the terminal parts of the reproductive system as the main basis for the systematic arrangement of the proterodiplostomids was also not supported, although these characters are certainly useful for differentiation among genera and species. Our 28S analyses do not show clear association between well-supported proterodiplostomid clades and the structure of their terminal reproductive ducts (Figs 3, 4, 6). For instance, in both 28S analyses (Figs 2, 3) *Paraproterodiplostomum* n. g., which has a uterus that opens into genital atrium separately from a common male efferent duct, forms a clade with *Archaeodiplostomum*, *Neocrocodilicola* n. comb., *Polycotyle*, and *Pseudocrocodilicola*, which all have the ejaculatory duct, paraprostate and metraterm form a common duct. Likewise, members of *Cystodiplostomum*, which have a paraprostate that opens separate from the ejaculatory duct and metraterm, formed a clade with the *Proterodiplostomum* spp. (excluding *Pt. globulare* formally included in *Proterodiplostomum*; see discussion below) possessing a metraterm that opens separately from the ejaculatory duct and the paraprostate. Although, the level of paraprostate development and the arrangement of terminal ducts of the reproductive system do not seem to be useful for identifying subfamilies of proterodiplostomids, these features are definitely suitable for differentiation among genera. We therefore provide schematic diagrams of almost all proterodiplostomid genera based on the original illustrations. (Fig. 6).

Based on all previously available and new molecular as well as morphological data, we abandon the subfamily structure of the Proterodiplostomidae. This decision is reminiscent of

other large digenean families that traditionally had a complex taxonomic structure, e.g., the Cryptogonimidae Ward, 1917, the Echinostomatidae Looss, 1899, and the Dicrocoeliidae Looss, 1899. In all those cases, the increasing amount of phylogenetic data indicated lack of support for existing subfamilies, which resulted in the abandonment of the subfamilies in all three families (Miller & Cribb, 2008; Tkach et al., 2016, 2018). This allows us to look at the evolution and taxonomy of this group unobstructed the by the systematic schemes based on ambiguous characters with unclear evolutionary history and relative weight. We believe this will accelerate the development of a natural classification system of the Proterodiplostomidae, based on a combination of molecular phylogenetic data and better understood morphological criteria.

Revision and additional systematic changes at genus level

The status of *Pseudocrocodilicola*

Until now the genus *Pseudocrocodilicola* contained 2 species: *Ps. americaniense* (type-species) and *Ps. georgiana* (Byrd & Reiber, 1942; Dubois, 1979). These species did not form a monophyletic clade in any of our analyses. *Pseudocrocodilicola georgiana* clustered with *Po. ornata* (85% support) in the 28S analysis of the Proterodiplostomidae and appeared as a separate branch in the *cox*1 tree (Figs 3, 4), whereas *Ps. americaniense* consistently formed a poorly supported clade with *Ar. overstreeti* n. sp. in both 28S and *cox*1 analyses (Figs 3, 4).

Besides the low branch support in the phylogenetic analyses, species of *Archaeodiplostomum* and *Pseudocrocodilicola* have very significant morphological differences, definitely warranting their placement into separate genera. *Archaeodiplostomum* spp. are characterized by having a very large ventral sucker, a prosoma and opisthosoma of similar length, vitellarium distributed in both the prosoma, and the opisthosoma and an ejaculatory duct

that joins the paraprostate at its base. In contrast, members of *Pseudocrocodilicola* have a prosoma that is typically much longer than the opisthosoma, vitellarium primarily limited to the prosoma, and a muscular pouch surrounding the common duct. In addition, *Ps. americaniense* has an ejaculatory duct that joins the paraprostate at its middle and *Ps. georgiana* has an ejaculatory duct that joins the paraprostate at its proximal (anterior) end (Byrd & Reiber, 1942; Fig. 6).

The two species of *Pseudocrocodilicola* also have significant morphological differences beyond the position of the junction of the ejaculatory duct with the paraprostate. The vitellarium of *Ps. americaniense* does not extend anteriorly to the level of the ventral sucker, whereas the vitellarium of *Ps. georgiana* always extends anteriorly beyond the level of the ventral sucker. Additionally, the metraterm of *Ps. americaniense* joins the common male efferent duct some distance after it exits the paraprostate to form the common duct (similar to that in *Archaeodiplostomum* spp.), whereas the metraterm of *Ps. georgiana* joins the distal (posterior) end of the paraprostate to form the common duct (Fig. 6C, D; Byrd & Reiber, 1942).

Based on the phylogenetic position, genetic distances and the above morphological differences between the two *Pseudocrocodilicola* species, we believe *Ps. georgiana* needs to be transferred to a new genus. Therefore, we establish *Neocrocodilicola* n. g. with *Neocrocodilicola georgiana* n. comb. as the type- and only species. An amended diagnosis of *Pseudocrocodilicola* and diagnosis of *Neocrocodilicola* n. g. are provided below.

Pseudocrocodilicola Byrd et Reiber, 1942

Diagnosis (after Niewiadomska, 2002e, amended): Body distinctly bipartite; prosoma flattened, lanceolate, longer than cylindrical opisthosoma. Oral sucker smaller than ventral sucker.

Pseudosuckers absent. Ventral sucker situated in middle or anterior to middle of prosoma; holdfast organ rather small, oval, with median slit bordered by papillae. Pharynx similar in size to oral sucker; caeca reaching level of paraprostate. Gonads occupying most of opisthosoma; testes tandem; paraprostate small, muscular, ellipsoidal, surrounded by relatively few, large gland-cells. Ejaculatory duct joins paraprostate near its midpoint. Ovary pretesticular; oötype intertesticular. Vitellarium distributed throughout posterior two thirds of prosoma, anterior extent at level of or before ventral sucker. Metraterm joins common male efferent duct to form common duct surrounded by thick-walled muscular pouch and opening into genital atrium. Excretory pore terminal. In *Alligator mississippiensis*. Nearctic. Type-species: *Pseudocrocodilicola americaniense* Byrd et Rieber, 1942

Neocrocodilicola n. g. Tkach, Achatz et Pulis

Diagnosis: Body distinctly bipartite; prosoma flattened, lanceolate, longer than cylindrical opisthosoma. Oral sucker smaller than ventral sucker. Pseudosuckers absent. Ventral sucker situated in middle or anterior to middle of prosoma; holdfast organ rather small, oval, with median slit bordered by papillae. Pharynx similar in size to oral sucker; caeca reaching level of paraprostate. Gonads occupying most of opisthosoma; testes tandem; paraprostate small, muscular, ellipsoidal, surrounded by relatively few large gland cells. Ejaculatory duct joins proximal end of paraprostate. Ovary pretesticular; oötype intertesticular. Vitellarium distributed throughout posterior two thirds of prosoma, always extending anteriorly beyond ventral sucker, sometimes slightly extending into opisthosoma. Metraterm joins common male efferent duct to form common duct surrounded by thick-walled muscular pouch and opening into genital atrium.

Excretory pore terminal. In *Alligator mississippiensis*. Nearctic. Type-species: *Neocrocodilicola georgiana* (Byrd et Rieber, 1942) n. comb.

Remarks

Neocrocodilicola n. g. differs by 1.9–5.3% of nucleotide positions in 28S sequences and 10.4–21.7% of nucleotide positions in *cox*1sequences from all other genera with available DNA sequence data.

Neocrocodilicola n. g. can be differentiated from *Heterodiplostomum* by the lack of a muscular pouch surrounding the paraprostate (Fig. 6D, S). *Neocrocodilicola* n. g. also lacks a muscular pouch enclosing the paraprostate, ejaculatory duct and metraterm found in *Capsulodiplostomum*. Unlike *Mesodiplostomum* and *Proalarioides, Neocrocodilicola* n. g. has a defined paraprostate (Fig. 6D, T, U). *Neocrocodilicola* n. g. has a relatively much smaller holdfast organ compared to *Ophiodiplostomum*, in which it occupies approximately half of the prosoma.

The metraterm of *Neocrocodilicola* n. g. joins the common male efferent duct to form the common duct. In contrast, *Proterodiplostomum*, *Pseudoneodiplostomum* and *Paraproterodiplostomum* n. g. possess a metraterm which opens separately from the male ducts (Fig. 6D, G, H, J, K). The ejaculatory duct of *Neocrocodilicola* n. g. joins the proximal half of the paraprostate (Fig. 6D), while in *Cystodiplostomum*, *Herpetodiplostomum*, *Massoprostatum*, *Paradiplostomum* and *Prolecithodiplostomum* the paraprostate, the ejaculatory duct and the metraterm open separately into the genital atrium (Fig. 6M, N, O, R).

Neocrocodilicola n. g. can be easily differentiated from *Polycotyle*, *Crocodilicola* and *Archaeodiplostomum* by the presence of a muscular pouch surrounding the common duct.

Furthermore, *Neocrocodilicola* n. g. does not possess small suckers along the opisthosoma, which are characteristic of *Polycotyle*. The vitellarium in *Neocrocodilicola* n. g. is not limited to the area around the holdfast organ as in *Crocodilicola*.

The ejaculatory duct in *Neocrocodilicola* n. g. joins the near the proximal end of the paraprostate, whereas in *Archaeodiplostomum* and *Pseudocrocodilicola* it joins either the common efferent male duct or the distal end of the paraprostate, respectively (Fig. 6B–D).

The status of *Proterodiplostomum*

Proterodiplostomum at present includes 6 species and is the most speciose genus of proterodiplostomids in the Neotropics. All known species have an ejaculatory duct and paraprostate that open side by side or with a common pore (without a common male efferent duct) and a metraterm which opens separately from the male ducts (Dubois, 1979; Catto & Amato, 1994). Two *Proterodiplostomum* species from caimans (the type-species *Pr. longum* and *Pr. tumidulum*) were described with a sucker-like muscular structure in the genital atrium, whereas *Pr. medusae* is known to have muscular bundles which are almost sucker-like in the wall of the genital atrium (Dubois, 1936a; Catto & Amato, 1994) (Fig. 6J, K). At the same time, *Pr. breve* and *Pr. globulare* (Fig. 6L) from caimans, and *Proterodiplostomum ophidum* Thatcher, 1963 from a snake were described without any sucker-like or muscular structures near the genital atrium (Thatcher, 1963; Catto & Amato, 1994).

In this study, we collected *Pr. longum, Pr. globulare*, *Pr. medusae* and an immature *Proterodiplostomum* species. Our specimens of *Pr. longum* have a well-defined sucker-like muscular structure in the genital atrium, whereas our specimens of *Pr. medusae* and the immature *Proterodiplostomum* sp. had well pronounced muscle bundles in the wall of the genital

atrium, which were almost sucker-like. In contrast, our specimens of *Pr. globulare* lacked any sucker-like or muscular structure in the wall of the genital atrium.

Our phylogenetic analyses revealed *Proterodiplostomum* to be non-monophyletic. *Proterodiplostomum longum* (type-species), *Pr. medusae* and an immature *Proterodiplostomum* sp. formed a strongly supported clade in our analyses (Figs 2–4). These three species have a sucker-like structure or well-defined muscle bundles in the wall of the genital atrium. In contrast, *Pr. globulare,* which lacks the sucker-like structure in the genital atrium, formed one of the branches in the basal polytomy within the Proterodiplostomidae in all our analyses (Figs 2–4). *Proterodiplostomum globulare* also showed 5.4–6.1% (59–67 bases) divergence in 28S sequences and significant 22.1–23.7% (116–122 bases) divergence in *cox*1 sequences from other *Proterodiplostomum* species in our study.

Based on the absence of a sucker-like structure in the genital atrium of *Pr. globulare* along with the strong phylogenetic evidence, we erect the genus *Proteroduboisia* n. g. for *Pr. globulare*.

Proteroduboisia n. g. Tkach, Achatz et Melo

Diagnosis: Body bipartite; prosoma elliptic, folicaeous; opisthosoma elongate, cylindrical. Oral and ventral suckers moderately developed; holdfast organ elliptical or subspherical, with papillae on margin of median slit. Pseudosuckers absent. Pharynx moderately developed; caeca reaching near level of genital atrium. Testes tandem; paraprostate relatively small; ejaculatory duct and efferent duct of paraprostate open together at apex of genital cone. Ovary pretesticular. Vitellarium extends from below or at level of ventral sucker to posterior margin of anterior testis or near posterior end of body. Metraterm opens separately from male ducts into genital atrium.

Genital atrium subterminal with dorsal opening. In caimans. Neotropics. Type-species: *Proteroduboisia globulare* (Catto et Amato, 1994) n. comb. Other species: *Proteroduboisia breve* (Catto et Amato, 1994) n. comb., *Proteroduboisia ophidum* (Thatcher, 1963) n. comb.

Remarks

Proteroduboisia n. g. differs by 3.2–6.1% of nucleotide positions in 28S sequences and 20.2–22.3% of nucleotide positions in *cox*1sequences from all other genera with available DNA sequence data.

Proteroduboisia n. g. can be easily morphologically differentiated from *Heterodiplostomum* and *Capsulodiplostomum* by the lack of a muscular pouch surrounding the paraprostate in *Heterodiplostomum* (Fig. 6L, S) or the paraprostate, ejaculatory duct and metraterm in *Capsulodiplostomum* (not shown on Fig. 6 due to the very poor quality of the illustration in the original description). Although relatively small, the paraprostate of *Proteroduboisia* n. g. is well-defined compared to the apparent lack of the paraprostate in *Mesodiplostomum* and *Proalarioides* (Fig. 6L, T, U). The holdfast organ in *Proteroduboisia* n. g. occupies approximately a quarter or less of the prosoma length, whereas in *Ophiodiplostomum* the holdfast organ occupies approximately half of the prosoma length. *Proteroduboisia* n. g. can be differentiated from most other proterodiplostomid genera based on the topology of the terminal reproductive ducts. The ejaculatory duct and efferent duct of the paraprostate open side by side in *Proteroduboisia* n. g. without forming a common male efferent duct, while the metraterm opens separately. The ejaculatory duct, paraprostate and metraterm unite in different ways to form a common duct in *Archaeodiplostomum*, *Crocodilicola*, *Neocrocodilicola*, *Polycotyle*, and *Pseudocrocodilicola*, whereas the paraprostate of *Cheloniodiplostomum*,

Cystodiplostomum, *Herpetodiplostomum*, *Paradiplostomum*, and *Prolecithodiplostomum* opens distinctly separately from the ejaculatory duct. In *Pseudoneodiplostomum* and *Paraproterodiplostomum* n. g. the ejaculatory duct joins the paraprostate (Fig. 6G, H). Morphological differences between *Proteroduboisia* n. g. and *Proterodiplostomum* are already discussed above.

Due to the erection of *Proteroduboisia* n. g. and transfer of 3 species into the new genus we provide an amended diagnosis of *Proterodiplostomum*.

Proterodiplostomum Dubois, 1936

Diagnosis (after Niewiadomska, 2002e, amended): Body distinctly bipartite; prosoma flattened, spatulate, typically much shorter than cylindrical opisthosoma. Oral sucker and ventral sucker moderately developed; holdfast organ elliptical, elongate, with papillae on margin of median slit. Pseudosuckers absent. Pharynx small or moderately developed; caeca reaching near level of genital atrium. Testes tandem; anterior testis near middle of opisthosoma. Paraprostate well-developed, tubular, reaching close to posterior testis. Ejaculatory duct and efferent duct of the paraprostate open together at apex of genital cone. Ovary pretesticular; oötype intertesticular. Vitellarium distributed throughout prosoma and opisthosoma, anterior extent before or after ventral sucker, posterior extent reaching about level of paraprostate. Metraterm opens separately from male ducts near base of genital cone. Muscular sucker-like structure or denser musculature present in wall of genital atrium. Genital atrium with subterminal opening, on dorsal side. Excretory pore terminal. In crocodilians. Neotropics. Type-species: *Proterodiplostomum longum* (Brandes, 1888). Other species: *Proterodiplostomum tumidulum* Dubois, 1936, *Proterodiplostomum medusae* (Dubois, 1936).

Status of *Pseudoneodiplostomoides*

Prior to this study, no members of the Proterodiplostomidae had been reported from Australian crocodilians. Two members of *Pseudoneodiplostomoides*, a previously-accepted subgenus of *Pseudoneodiplostomum*, were described by Tubangui & Masiluñgan (1936) and Yamaguti (1954) from saltwater crocodile *Crocodylus porosus* Schneider from the Philippines and Indonesia, respectively. Tubangui & Masiluñgan (1936) originally placed their species (*Pu. crocodilarum*) from the Philippines within the genus *Neodiplostomum* Railliet, 1919. Yamaguti (1954) later established the subgenus *Pseudoneodiplostomoides* for his newly described *Pseudoneodiplostomum* (*Pseudoneodiplostomoides*) *crocodili* Yamaguti, 1954 and *Pu. crocodilarum*, in part based on the presence of 2 muscular pits in the genital atrium. Dubois (1979) listed both of these species as synonyms of *Pe. siamense*. We disagree with Dubois' synonymization because of the lack of the characteristic "pits" or concave invaginations of the genital atrium wall in *Pe. siamense*, but their presence in the members of Yamaguti's subgenus *Pseudoneodiplostomoides*. Our molecular data support this notion with 1.4 % (15 bases) divergence between *Pe. siamense* and *Pu. crocodilarum* in the 28S gene and 17.7% (92 bases) divergence in *cox*1. Considering the substantial level of sequence divergence, the results of our phylogenetic analyses (Figs 2–4) and the lack of the characteristic invaginations in the genital atrium of all other *Pseudoneodiplostomum* species, including our specimens representing 4 species, we restore the *Pseudoneodiplostomoides* and elevate it to genus level. Since the only character Yamaguti (1954) used to separate *Pu. crocodili* and *Pu. crocodilarum* was the width of the eggs, we consider *Pu. crocodili* a junior synonym of *Pu. crocodilarum* (Tubangui & Masiluñgan, 1936) n. comb. which becomes the type-species of *Pseudoneodiplostomoides*.

Yamaguti (1954) provided an adequate diagnosis of *Pseudoneodiplostomoides*, therefore we do not include an amended diagnosis here.

Content of *Pseudoneodiplostomum*

Pseudoneodiplostomum includes 4 currently accepted species: *Pe. thomasi* (type-species) and *Pe. bifurcatum* from Africa, *Pe. siamense* from Southeast Asia, and *Pe. groschafti* from Cuba (Dubois, 1979; Moravec, 2001). *Pseudoneodiplostomum thomasi* was originally described by Dollfus (1935) from the intestine of the dwarf crocodile *Osteolaemus tetraspis* Cope collected in the French Congo. Dubois (1948) examined specimens collected from the intestine of the West African slender-snouted crocodile *Mecistops cataphractus* (Cuvier) collected in Gabon that were previously identified as *Pe. thomasi*. Based on these specimens, Dubois (1948) established the subspecies *Pe. thomasi gabonicum* Dubois, 1948, which differed from the nominal subspecies *Pe. thomasi thomasi* by the greater opisthosoma:prosoma ratio, narrower body, as well as the smaller ventral sucker, holdfast organ, ovary and testes.

Our specimens of *Pe. thomasi thomasi* and *Pe. thomasi gabonicum* differ by 0.2% of 28S sequences. For comparison, 28S sequences of *Pe. bifurcatum* and *Pe. thomasi* were identical despite the two species having very distinct morphologies. Based on the morphological and genetic differences we elevate *Pe. thomasi gabonicum* to species level as *Pe. gabonicum* Dubois, 1948 n. nom.

Notes on other genera

Crocodilicola pseudostoma was originally described from *Al. mississippiensis* collected in South Carolina, U.S.A. (Willemoes-Suhm, 1870) and later reported from the same host in

several locations in the United States, as well as from Morelet's crocodile *Co. moreletii* in Mexico (Tellez, 2014). Our analyses of 28S and *cox*1 included sequences of *Cr. pseudostoma* from GenBank published by Hernández-Mena et al. (2017). These sequences came from a metacercaria collected from fish in Catemaco Lake, Veracruz, southern Mexico, thousands of kilometers from the type territory of *Cr. pseudostoma* or the nearest current area populated by alligators. In our phylogenetic analyses, these sequences formed strongly supported clades with proterodiplostomids from caimans collected in Brazil (Figs 2–4). The distribution of *Co. moreletii* overlaps with that of caimans, but not with the range of the American alligator. All proterodiplostomids from *Al. mississippiensis* included in our analyses, formed a strongly supported monophyletic group and all other genera of proterodiplostomids parasitizing crocodilians showed close association with a single genus of their definitive hosts (Fig. 3).

The combination of the definitive host distribution patterns and the phylogenetic placement of *Cr. pseudostoma* sequences from GenBank suggests that the identification of those metacercariae should be considered with caution. Although *Cr. pseudostoma* was reported from *Co. moreletii* in Mexico (Dubois, 1953, Thatcher, 1964), we believe these reports were a result of misidentification due to the poor condition of the specimens. We examined specimens of *Cr. pseudostoma* from *Co. moreletii* in Mexico deposited in the HWML (see Materials & Methods). Despite the very poor state of the specimens on slides it was evident that they do not belong to *Crocodilicola*. The ejaculatory duct in those specimens joins the paraprostate near its proximal end and the metraterm clearly does not join the merged ejaculatory duct and paraprostate. In *Cr. pseudostoma* the metraterm, ejaculatory duct and paraprostate merge to form a common duct. Most likely, these specimens represent a new species, however, their state does not allow for a quality description. Sequencing of an adult stage of *Cr. pseudostoma* from alligators as well as of

proterodiplostomids from *Co. moreletii* in the future will eventually support or reject the identification of the metacercariae from Hernández-Mena et al. (2017). We anticipate that it will turn out to be a new genus, possibly shared between caimans and crocodiles in the Neotropics.

Cystodiplostomum hollyi is the type-species of the monotypic genus *Cystodiplostomum*. Our unidentified *Cystodiplostomum* sp. formed 100% supported clades with *Cy. hollyi* in both 28S and *cox*1 analyses. It most likely represents a second member of the genus, but our only specimen was used for DNA extraction. Due to the relatively high level of genetic divergence, it is also possible that our specimen represents another genus not available for our analysis, such as *Prolecithodiplostomum*, in which the topology of terminal reproductive ducts is identical to that of *Cystodiplostomum* (Fig. 6M).

Unlike other proterodiplostomid taxa included in our study, adult *Heterodiplostomum* are parasites of the intestines of snakes in the Neotropics and are known to use amphibians as second intermediate hosts (Niewiadomska, 2002e; Queiroz et al., 2020). At present, *Heterodiplostomum* includes two species: *He. lanceolatum* and *Heterodiplostomum helicopsis* Mañé-Garzón et Alonso, 1976. Ribosomal sequences from metacercaria of *He. lanceolatum* collected from pointed belly frogs *Leptodactylus podicipinus* (Cope) in Brazil were recently published (Queiroz et al., 2020) and differ from our sequences of *Heterodiplostomum lanceolatum* by 0.2% (2 nucleotides). No *cox*1 sequences are available for the previously published *He. lanceolatum* isolate. *Heterodiplostomum lanceolatum* has been described with caeca that terminate anterior to the copulatory bursa and vitellarium that do not extend anterior passed the holdfast organ; whereas the caeca of *He. helicopsis* terminate near the distal extremity of the opisthosoma and the vitellarium extend anteriorly to near the level of the caecal fork (Dubois, 1936a; Mañé-Garzón & Alonso, 1976). Whereas our specimens of *Heterodiplostomum* from *L. chaquensis* and

E. poecilogyrus were immature, they had morphology corresponding to the description of *He. lanceolatum* (i.e., caeca terminate immediately anterior to the copulatory bursa and vitellarium does not pass the level of the holdfast organ). We suspect that the difference in the 28S gene sequences may be indicative of the presence of a cryptic species. None of the remaining proterodiplostomids included in this study had more than a single variable nucleotide site within a species and *Pe. bifurcatum* and *Pe. thomasi* had no differences in their 28S sequences. However, future studies will have to include sequences from adult *Heterodiplostomum* specimens along with sequences of faster mutating genes (e.g., *cox*1) to properly test for the presence of cryptic species within this genus.

As a result of the present revision of several proterodiplostomid taxa and abandonment of the subfamilies within the Proterodiplostomidae the family now includes 21 genera. We expect additional changes in the system of this family as our knowledge of proterodiplostomid diversity and morphology, as well as greater sequencing coverage, will continue to improve with further studies. Nevertheless, we consider it useful to provide a key to the identification of the currently recognized proterodiplostomid genera. Although we do not believe that hosts or geographical distribution should be used as characters in the identification, we provide this information in the key for convenience.

Key to the genera of the Proterodiplostomidae

1a. Paraprostate absent **2**

1b. Paraprostate present **3**

2a. Ejaculatory duct and metraterm merge to form hermaphroditic duct near apex of genital cone. Hermaphroditic duct not enclosed in a muscular pouch. Pseudosuckers absent. In crocodilians. Neotropics ..... *Mesodiplostomum*

2b. Ejaculatory duct and metraterm merge to form hermaphroditic duct enclosed in a muscular pouch. Pseudosuckers present. In snakes. Palaeartic and Orient *Proalarioides*

3a. Paraprostate surrounded by muscular pouch. Paraprostate duct eversible. Ejaculatory duct and metraterm open side by side. In snakes. Neotropics

..... *Heterodiplostomum*

3b. Paraprostate not surrounded by muscular pouch or all terminal ducts of reproductive system enclosed in single muscular pouch **4**

4a. Entire paraprostate, ejaculatory duct, and metraterm enclosed in muscular pouch. Ejaculatory duct and metraterm open separately. In crocodilians. India .....

..... *Capsulodiplostomum*

4b. Paraprostate, ejaculatory duct, and metraterm not enclosed in muscular pouch. Ejaculatory duct and metraterm open separately or have a common opening **5**

5a. Vitellarium confined to opisthosoma. In crocodilians. Neotropics

..... *Massoprostatum*

5b. Vitellarium distributed differently **6**

6a. Holdfast organ relatively massive, typically occupying approximately half of prosoma

..... **7**

6b. Holdfast organ not as massive, typically occupying approximately 25–30% of prosoma

..... **8**

7a. Ejaculatory duct joins distal part of paraprostate. Two muscular pits, occasionally sucker-like, present in wall of genital atrium. In crocodilians. Australasia .....

.....*Pseudoneodiplostomoides*

7b. Ejaculatory duct and paraprostate do not join/unite. Muscular pits in wall of genital atrium absent. In snakes. Neotropics ..... *Ophiodiplostomum*

8a. Metraterm, ejaculatory duct and paraprostate join to form a common duct or all three share common opening **9**

8b. Metraterm, ejaculatory duct and paraprostate do not form common duct. Ejaculatory duct and paraprostate or ejaculatory duct and metraterm may join or share common opening

..... **13**

9a. Opisthosoma with longitudinal row of sucker-like structures on dorsal side. In crocodilians. Nearctic ..... *Polycotyle*

9b. Opisthosoma without dorsal sucker-like structures **10**

10a. Terminal part of paraprostate, ejaculatory duct, and metraterm enclosed in muscular pouch

..... **11**

10b. Paraprostate, ejaculatory duct, and metraterm not enclosed in muscular pouch **12**

11a. Ejaculatory duct typically joins paraprostate near its midpoint. In crocodilians. Nearctic

..... *Pseudocrocodilicola*

11b. Ejaculatory duct joins paraprostate near its proximal end. In crocodilians. Nearctic.

..... *Neocrocodilicola* n. g.

12a. Vitelline follicles confined to area around holdfast organ. Separation between prosoma and opisthosoma indistinct. In crocodilians. Nearctic and Neotropics.

……..... *Crocodilicola*

12b. Vitelline follicles distributed in both prosoma and opisthosoma, extending well beyond area around holdfast organ. Separation between prosoma and opisthosoma distinct. In crocodilians. Nearctic ..... *Archaeodiplostomum*

13a. Paraprostate opens separately from ejaculatory duct and metraterm. Ejaculatory duct and metraterm may join or share common opening **14**

13b. Metraterm opens separately from ejaculatory duct and paraprostate. Ejaculatory duct and paraprostate may join or share common opening **18**

14a. Genital cone present **15**

14b. Genital cone absent **17**

15a. Genital cone massive, equal to about 1/4 of total body length. In crocodilians. Neotropics ..... *Paradiplostomum*

15b. Genital cone much smaller, not more than 1/8 of total body length **16**

16a. Holdfast organ with papillae. In crocodilians. Neotropics ..... *Herpetodiplostomum*

16b. Holdfast organ without papillae. In chelonians. Neotropics .... *Cheloniodiplostomum*

17a. Thick-walled, sucker-like dorsal invagination of body present near midpoint of opisthosoma or slightly more posterior. In crocodilians. Neotropics .... *Cystodiplostomum*

17b. No thick-walled, sucker-like dorsal invagination of body present. In crocodilians. Neotropics ..... *Prolecithodiplostomum*

18a. Sucker-like muscular structure (well-developed or concentrated muscle bundles) in the wall of the genital atrium present. In crocodilians. Neotropics .... *Proterodiplostomum*

18b. Genital atrium without sucker-like structure **19**

19a. Ejaculatory duct does not join paraprostate. Ejaculatory duct and paraprostate share common opening. In crocodilians and snakes. Neotropics ..... *Proteroduboisia* n. g.

19b. Ejaculatory duct joins paraprostate **20**

20a. Ejaculatory duct joins paraprostate near its distal end. Paraprostate well-developed. In crocodilians. Nearctic ..... *Paraproterodiplostomum* n. g.

20b. Ejaculatory duct joins proximal half of paraprostate. Paraprostate weakly developed. In crocodilians. Africa, Australasia and Neotropics ..... *Pseudoneodiplostomum*

Host and geographic associations

The Proterodiplostomidae clearly is a very old evolutionary lineage of digeneans parasitizing an ancient group of hosts that already existed and strongly radiated before the separation and subsequent drift of the current continents. In a series of works, Brooks and co-authors (Brooks, 1979; Brooks & O'Grady, 1989; Brooks et al., 1992) presented morphology-based phylogenies of the Proterodiplostomidae (along with some other digenean groups parasitic in crocodilians) and analyzed their historical biogeography as well as host associations. These authors emphasized that the history of proterodiplostomid associations with their crocodilian hosts extended deep into the geological and evolutionary past and was affected by major global geological events such as tectonic plate movement and accompanying radiation among and within the crocodilian lineages.

Our phylogenetic analyses supported some of the conclusions drawn in these publications, e.g., regarding the monophyly of the proterodiplostomids parasitizing alligators. The arrangement of the remaining taxa showed, however, a substantial disagreement. Although our 28S tree was not fully resolved (Fig. 3) it provides some new insights into the historical biogeography and host associations of the Proterodiplostomidae. This is particularly interesting considering the recent advances in the phylogenetics of crocodilians and discovery of cryptic

species based on both morphological and molecular criteria (e.g., Brochu 1997, 2003; Bittencourt et al., 2019; Brochu & Sumrall 2020; Roberto et al., 2020). Molecular data also suggested a relatively recent radiation and active speciation of the true crocodiles (Oaks, 2011).

Although morphology based phylogenetic hypotheses incorporating both the extant and extinct species suggested that gharials (Gavialidae Adams) represent the most basal lineage of extant crocodilians (Brochu, 1997, 2003), the molecular phylogenies (Oaks, 2011) strongly suggested that the Alligatoridae Gray is the basal extant group. Although our 28S phylogeny was not completely resolved, the interrelationships between proterodiplostomids correspond well to the phylogenetic affinities among crocodilians (Figs 3, 8).

According to our data, the proterodiplostomids of alligatorids are not monophyletic (but those parasitic in American alligators are) because the genera associated with caimans are found in 3 different clades. This tree topology allows us to hypothesize that the proterodiplostomids parasitic in crocodilians have first evolved and radiated into several lineages in the ancestors of modern caimans yet in Pangea. Some of these lineages were either inherited by alligators and then true crocodiles in the process of crocodilian radiation or passed as a result of subsequent host switching events. This hypothesis corresponds with at least some of the previously suggested schemes of the biogeographic relationships among crocodilians (Sill, 1968; Brooks, 1979). The evidence of at least one genus (*Pseudoneodiplostomum*) shared between members of the genus *Crocodylus* in Africa and Australia (and Asia according to published morphology-only data) fits well the hypothesis of relatively recent active radiation of *Crocodylus* (Oaks, 2011; Figs 3, 8). The close relationships between clades uniting parasites of *Alligator* and *Crocodylus* suggests that as true crocodiles radiated they likely received their original proterodiplostomids

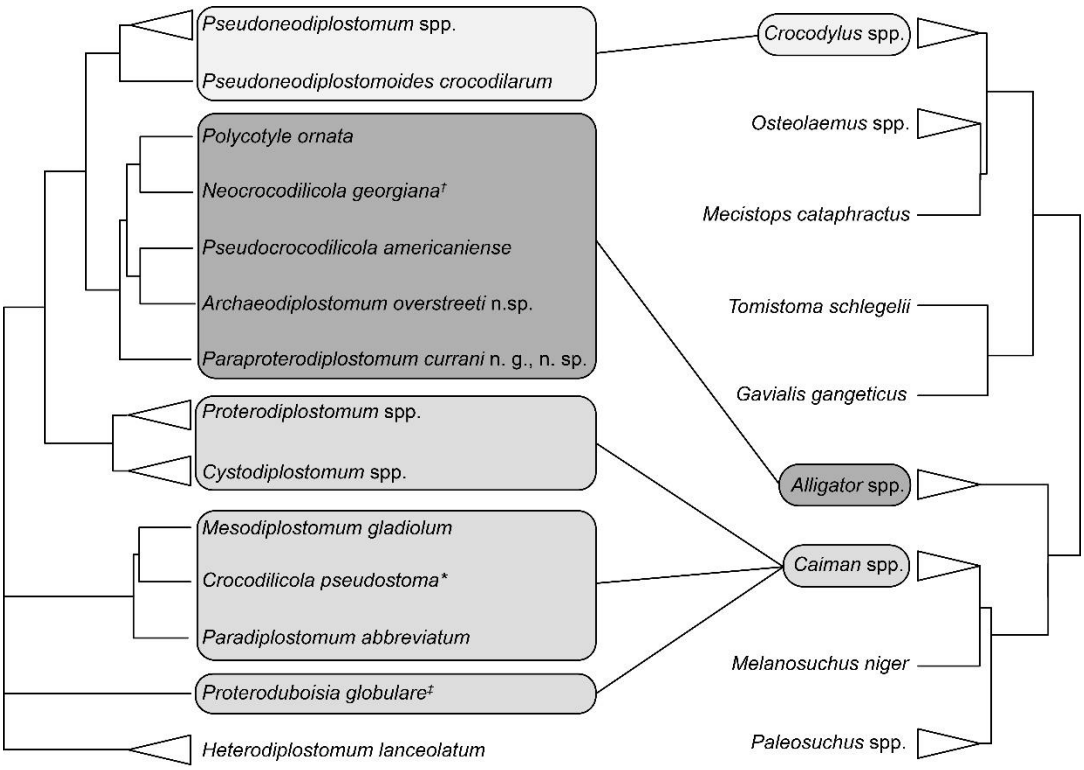

Figure 8. Phylogenetic tree of the Proterodiplostomidae from the present study and the molecular phylogenetic tree of the Crocodilia (modified from Oaks, 2011) showing host associations between currently sequenced proterodiplostomids and extant crocodilian linages. Phylogenetic trees are presented as rectangular cladograms for convenience. Connecting lines and identical shades of grey color indicate host associations. (after Tkach et al., 2020)

from ancestors of modern alligators. It is difficult to speculate, however, where this could have happened geographically due to the broad distribution of both crocodilian lineages in the past.

Despite the paraphyly shown by the proterodiplostomids parasitic in caimans, all sub-clades in our tree (Figs 3, 8) demonstrated strong associations between genera of proterodiplostomids and crocodilians. Proterodiplostomids from *Alligator* and *Crocodylus* formed well-supported monophyletic clades despite the high level of morphological diversification among members of each clade. The only deviation from the strict specificity to host genera in monophyletic clades in the 28S tree is the position of *Cr. pseudostoma* (GenBank MF398328) together with *Paradiplostomum* and *Mesodiplostomum*, parasites of caimans (Fig.

3). As explained above, we believe that the sequences deposited in GenBank as *Cr. pseudostoma* were obtained from erroneously identified metacercariae.

Missing taxa and future prospects

Despite our extensive sampling effort and the broad representation of proterodiplostomid taxa in the resulting dataset, some critically important taxa and sequences from them are still missing. There are several crocodilian species in Asia, Africa, South and Central America that have not been examined for proterodiplostomids at all. Some of them are endemic to a single island or a limited geographic area and therefore may have endemic parasite fauna. On the other hand, some crocodilians including different species of caimans are known as hosts of a diverse proterodiplostomid fauna, which have not been a subject of molecular systematic and phylogenetic analyses.

Some of the gaps are, however, more glaring than others. Probably the biggest gap in the available data is the lack of sequences from any proterodiplostomids parasitizing gharials, which were repeatedly considered the basal group of extant crocodilians in morphology-based analyses. In addition, the distribution area of gharials lies within the overall distribution of the genus *Crocodylus* and overlaps with the current or recent historical distribution of mugger crocodile *Crocodylus palustris* (Lesson) and *Co. porosus*. Equally missing and extremely interesting are proterodiplostomids from the Chinese alligator *Alligator sinensis* Fauvel, now critically endangered and on the brink of extinction. Therefore, only fixed museum specimens may potentially be a source of parasite samples. Other crucial hosts are American crocodile *Crocodylus acutus* Cuvier, Orinoco crocodile *Crocodylus intermedius* Graves, and *Co. moreletii* whose geographic ranges overlap with the distribution areas of the American alligator and

several species of caimans. Obtaining sequence data from proterodiplostomids parasitic in these hosts may potentially answer a variety of enticing questions regarding their evolutionary origin as well as the extent of physiological vs ecological specificity to their hosts. In addition to proterodiplostomids from crocodilian hosts, several known taxa of these digeneans parasitic in other hosts, such as snakes and turtles, are also awaiting sequencing and inclusion in future phylogenetic analyses.

Nevertheless, despite the lack of some important proterodiplostomids taxa in our analysis we believe that the views on their interrelationships and systematics presented here are well supported. Denser taxonomic sampling from a greater diversity of hosts and additional geographic areas should provide a solid background for a better understanding of the Proterodiplostomidae and their evolution and address the remaining unanswered questions presented in this study.

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Associated publicaiton

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CHAPTER V

PHYLOGENETIC AFFINITIES OF UVULIFER SPP. (DIGENEA: DIPLOSTOMIDAE) IN THE AMERICAS WITH DESCRIPTION OF TWO NEW SPECIES FROM PERUVIAN AMAZON

Introduction

The digenean genus *Uvulifer* Yamaguti, 1934 (Diplostomidae: Crassiphialinae) contains between 16 and 19 species worldwide, with the majority of the species parasitic in kingfishers (see Dubois, 1964; Yamaguti, 1971; Subair et al., 2013). The known life cycles for species of *Uvulifer* have a *Neascus*-type metacercaria that encysts on an aquatic vertebrate intermediate host, normally a fish (Hunter, 1933; Niewiadomska, 2002d). Often, the metacercariae become melanized by the fish host, which manifests as 'black spot' disease (Niewiadomska, 2002d; McAllister et al., 2013). Prior to this study, 6 valid species of *Uvulifer* were recognized from the Americas. Two of these species are distributed only in the Nearctic, 3 are distributed only in the Neotropics, and 1 species is distributed in both the Nearctic and Neotropics (Dubois, 1938, 1985, 1988; Muzzall et al., 2011; López-Jiménez et al., 2018). *Uvulifer ambloplitis* (Hughes, 1927) and *Uvulifer semicircumcisus* Dubois et Rausch, 1950 infect the belted kingfisher, *Megaceryle alcyon* (Linnaeus), in North America (Hunter, 1933; Dubois & Rausch, 1950). *Uvulifer prosocotyle* (Lutz, 1928) was reported from the ringed kingfisher, *Megaceryle torquata* Linnaeus, in Brazil and the Amazon kingfisher, *Chloroceryle amazona* (Latham), in Venezuela (Dubois, 1938; Caballero & Diaz-Ungria, 1958). *Uvulifer weberi* Dubois, 1985 is known from *C. amazona*, the green kingfisher, *Chloroceryle americana* (Gmelin), and the green-and-rufous

kingfisher, *Chloroceryle inda* (Linnaeus), in Paraguay (Dubois, 1985, 1988). *Uvulifer elongatus* Dubois, 1988 was described from *M. torquata* in Paraguay (Dubois, 1988), and *Uvulifer spinatus* López-Jiménez, Pérez-Ponce de León, et García-Varela, 2018 was recently described from *C. americana* in Mexico and also found in Guatemala, Honduras and Nicaragua (López-Jiménez et al., 2018).

In the present study, we describe 2 previously unknown species of *Uvulifer* from *C. inda* in the Cordillera Azul National Park, Peruvian Amazon. We generated partial sequences of the nuclear large subunit ribosomal RNA gene (28S) and the mitochondrial cytochrome oxidase 1 gene (*cox*1) from both new species and 5 additional species of *Uvulifer* collected from various kingfishers from South and North America and a fish from North America. Newly generated sequences were aligned and compared, and observed differences were used for augmenting morphological comparisons among species. Phylogenetic analyses were conducted independently for both gene fragments using new sequence data plus available congeneric sequence data from GenBank.

Materials & Methods

Specimens

Adult specimens belonging to the genus *Uvulifer* were obtained from *C. inda* collected in the Cordillera Azul National Park, Peru, *C. americana* and *M. torquata* from Pantanal, Fazenda Retiro Novo, Municipality of Poconé, Mato Grosso State, Brazil, *M. torquata* from the vicinities of Lábrea, State of Amazonas, Brazil and *M. alcyon* from Minnesota. In addition, a metacercaria of *Uvulifer* sp. was collected from a yellow perch, *Perca flavescens* Mitchill, from Minnesota (Table 6).

Phylogenetic analyses

Phylogenetic interrelationships among members of *Uvulifer* were analyzed using 28S and *cox*1 datasets as separate alignments. Newly obtained and previously published sequences were aligned with Clustal W (Larkin et al., 2007) as implemented in the BioEdit version 7.0.5.3 software (Hall, 1999); both alignments were trimmed to the length of the shortest respective sequence. *Ornithodiplostomum scardinii* (Shulman, 1952) was used as outgroup in the 28S analysis while *O. scardinii* and *Posthodiplostomum centrarchi* Hoffman, 1958 were used in *cox*1 analysis based on the topologies presented in the phylogenetic study by López-Jiménez et al. (2018).

The 28S alignment included newly generated sequences of 7 species of *Uvulifer* and previously published sequences of 6 species-level lineages of *Uvulifer*, only 1 of them (*U. spinatus*) representing an identified species. The *cox*1 alignment included newly generated sequences of 7 species of *Uvulifer* and a single previously published compatible sequence of *Uvulifer* sp. Additional *cox*1 sequences of *Uvulifer* available in GenBank were non-compatible with our sequences or were much shorter in length.

Independent phylogenetic analyses (separate 28S rRNA and *cox*1 gene alignments) were conducted using Bayesian Inference (BI) as implemented in MrBayes Ver. 3.2.6 software (Ronquist & Huelsenbeck, 2003). The general time-reversible model with estimates of invariant sites and gamma-distributed among-site variation (GTR + I + G) was identified as the best-fitting nucleotide substitution model for the 28S dataset using MEGA7 (Kumar et al., 2016). The Hasegawa-Kishino-Yano and gamma-distributed among-site variation (HKY + G) model was identified as the best-fitting nucleotide substitution model for each of the partitioned nucleotide codon position. BI analyses were performed using MrBayes software as follows: Markov chain

Table 6. List of *Uvulifer* species sequenced including their host species, geographical origin of material, morphological voucher numbers and GenBank accession numbers. HWML: Harold W. Manter Laboratory, University of Nebraska State Museum, Lincoln, NE, U.S.A.

Digenean taxa	Host species	Geographic origin	Museum No.	Accession numbers	
				28S	***cox*1**
Uvulifer ambloplitis	*Megaceryle alcyon*	U.S.A.	HWML-139982	MK874320	MK871329
Uvulifer batesi n. sp.	*Chloroceryle inda*	Peru	HWML-139983, HWML-139984	MK874321	MK871330
Uvulifer elongatus	*Megaceryle torquata*	Lábrea, Brazil	–	MK874322	MK871331
U. elongatus	*M. torquata*	Pantanal, Brazil	HWML-139985	MK874323	MK871332
Uvulifer pequenae n. sp.	*C. inda*	Peru	HWML-139986, HWML-139987	MK874324	MK871333
Uvulifer prosocotyle	*M. torquata*	Pantanal, Brazil	HWML-139988	MK874325	MK871334
Uvulifer weberi	*Chloroceryle americana*	Pantanal, Brazil	HWML-139989	MK874326	MK871335
Uvulifer sp.	*Perca flavescens*	U.S.A.	–	MK874327	MK871336

Monte Carlo (MCMC) chains were run for 3,000,000 generations with a sample frequency of 1,000, log-likelihood scores were plotted and only the final 75% of trees were used to produce the consensus trees by setting the "burn-in" parameter at 750. This number of generations was considered sufficient because the SD dropped below 0.01. The trees were visualized in FigTree ver. 1.4 software (Rambaut, 2016) and annotated in Adobe Illustrator®.

Results

Descriptions of new taxa

Uvulifer pequenae n. sp.

(Fig. 9)

Description [Based on 2 fully mature specimens]: Body 1,403–1,432 long, comprising a prosoma and opisthosoma; prosoma pyriform, ventrally concave, 480–517 long, with maximum width in the posterior half (304–318); opisthosoma elongated, 922–932 long and claviform with maximum width near midpoint (202–236). Prosoma: opisthosoma length ratio 0.54–0.57. Tegumental spines covering prosoma but limited to anterior 25% of opisthosoma. Oral sucker nearly terminal, 68–77 x 88–99. Prepharynx absent or not apparent. Pharynx oval, 45–56 x 34–37. Esophagus slightly longer than pharynx. Cecal bifurcation in anterior third of prosoma. Ceca slender, blind, extending to near posterior end of opisthosoma. Ventral sucker delicate, much smaller than oral sucker, 39–40 x 45–48, located at 60–62% of the prosoma length from the anterior end. Holdfast organ immediately posterior to ventral sucker (72% of the prosoma length from the anterior end); oval with ventral muscular portion having a longitudinal slit-like opening and basal glandular portion embedded in the prosoma, 133–136 x 99–114. Testes tandem, with smooth or slightly irregular margins, anterior testis 167–173 x 142–156, posterior testis 97–153 x

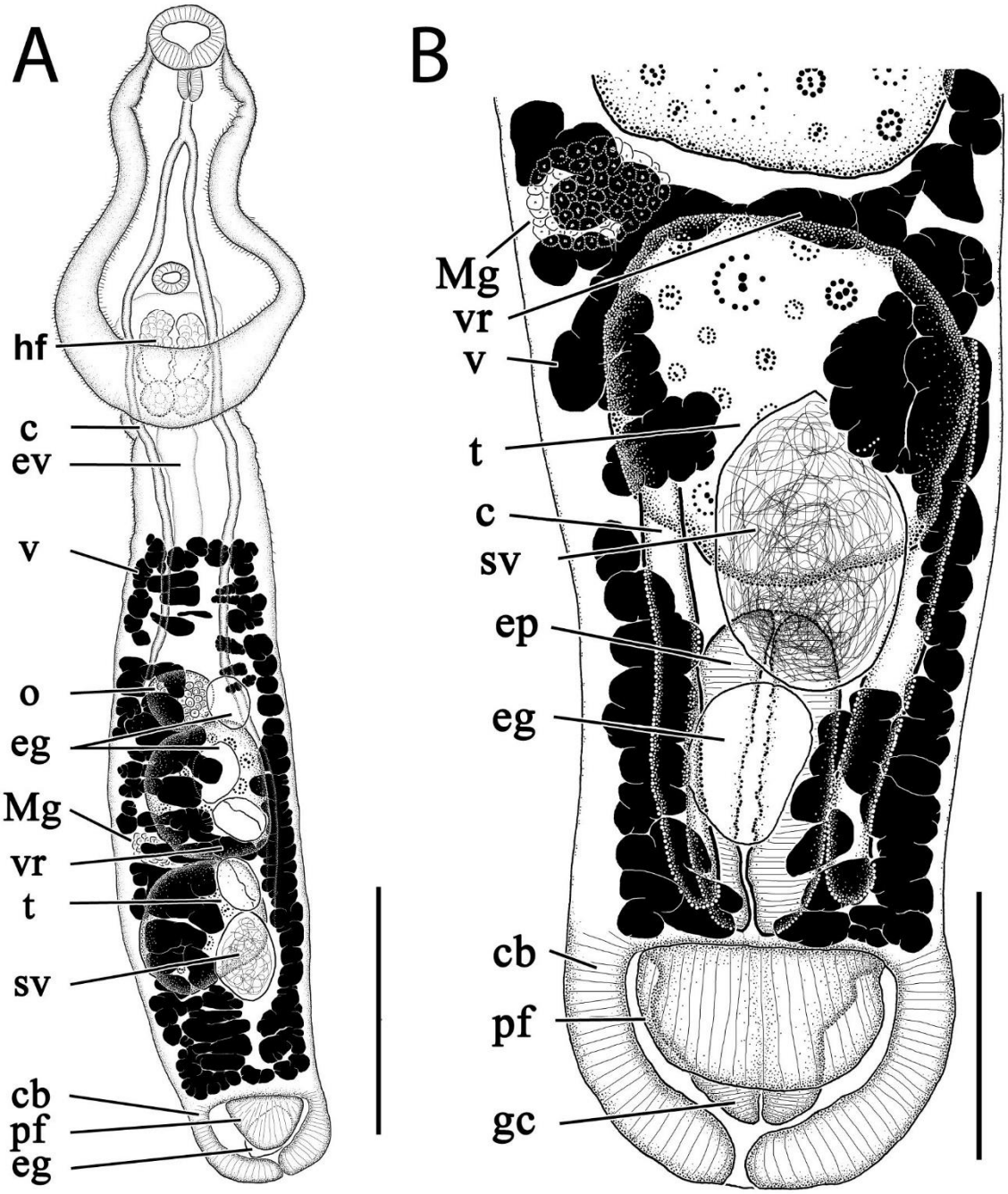

Figure 9. *Uvulifer pequenae* n. sp. **(A**) Ventral view of whole mount. (**B**) Ventral view of posterior body end. Scale bars: A, 300 µm; B, 100 µm. Abbreviations: c, ceca; cb, copulatory bursa; eg, egg; ep, ejaculatory pouch; ev, excretory vesicle; gc, genital cone; hf, holdfast organ; Mg, Mehlis'gland; o, ovary; pf, preputial fold; sv, seminal vesicle; t, testis; v, vitelline follicle; vr, vitelline reservoir. (after Achatz et al., 2019a)

77–82. Seminal vesicle subglobular, ventral to posterior testis, connected to ejaculatory duct; proximal ejaculatory duct tubular and running antero-dorsally, then bending and running posteriorly; distal portion opening into a muscular ejaculatory pouch. Ejaculatory pouch 142–156 x 71–85, draining posteriorly through narrow short male duct posteriorly; duct uniting with female system. Ovary submedian, (slightly dextral), immediately pretesticular (32% of the opisthosoma length from the anterior end), subspherical, 79–85 x 82–91. Ootype surrounded by Mehlis' gland, submedian, (slightly dextral), intertesticular. Seminal receptacle subspherical, immediately dorsal to ootype, smaller than ovary. Uterus ventral in opisthosoma, extending from ovarian level to posterior margin of posterior testis, containing from 2–5 eggs (71–81 x 46–57); distal uterus uniting with male duct and forming hermaphroditic canal; hermaphroditic canal descending into genital cone. Genital cone 60–65 x 94–97, extends into a bulbous copulatory bursa; copulatory bursa with muscular ventral preputial fold. Ventrolateral preputial lobe 45–65 x 82–94. Vitelline follicles located in opisthosoma, ventral and lateral to gonads, absent in the anterior 13–16% of the opisthosoma and posterior 11–12% of opisthosoma. Vitelline reservoir intertesticular, sinistral to ootype. Excretory vesicle I-shaped, with main stem dorsal in opisthosoma; stem ascending into prosoma and surrounding holdfast organ and giving rise to 6 longitudinal branches that extend toward oral sucker; branches interconnected by network of anastomosing channels throughout prosoma. Excretory pore not observed.

Taxonomic summary

Type host: Chloroceryle inda (Linnaeus) (Coraciiformes: Alcedinidae).

Site of infection: Small intestine.

Type locality: San Martín, Tocache Province, Cordillera Azul National Park, Río Pescadero, NE of Shapaja (8°10.694'S, 76°13.422'W), Peru, elev. 953 m above sea level.

Type specimens deposited: The type series consists of 2 fully mature specimens deposited in the Harold W. Manter Laboratory. Holotype: HWML 139986, labeled ex. *C. inda*, small intestine, Cordillera Azul National Park, Peru, 13 Nov 2013, coll. K. Patitucci; paratype: HWML 139987, label identical to the holotype. Symbiotype deposited in the Field Museum, Chicago (FMNH 3859910).

Representative DNA sequences: GenBank MK874324 (28S), MK871333 (*cox*1).

ZooBank registration: urn:lsid:zoobank.org:act:ED554410-BFDC-4FBD-AC4B-38A4BAF9213D

Etymology: The species is named after Tatiana Z. Pequeño Saco who provided invaluable assistance in organizing the field collecting in the Cordillera Azul.

Remarks

The new species clearly belongs to *Uvulifer* based on the combination of characteristic features that include the vitelline follicles confined to the opisthosoma, the presence of a muscular ejaculatory pouch, and a muscular copulatory bursa containing a retractile or protrusible genital cone partially surrounded by a ventrolateral preputial muscular fold (Niewiadomska, 2002d).

We believe only mature specimens of *Uvulifer* should be used for reliable morphological identification. *Uvulifer pequenae* is distinguishable from *U. elongatus*, *U. semicircumcisus*, *U. spinatus* and *U. weberi* by relatively shorter vitellarium. The vitellarium of all these 4 species occupies almost the whole length of the opisthosoma, whereas in *U. pequenae* it is absent in the

first 13–16% of the opisthosoma. The new species also differs from these 4 species by a greater prosoma: opisthosoma length ratio (see below).

Uvulifer pequenae can be further distinguished from *U. elongatus* by a much shorter body length (1,403–1,432 in the new species vs. 2,200–3,300 in *U. elongatus*), a much smaller ventral sucker (39–40 x 45–48 in the new species vs. 85–100 x 100–120 in *U. elongatus*) and slightly smaller eggs (71–81 in the new species vs. 80–90 in *U. elongatus*). The most dramatic difference between *U. pequenae* and *U. elongatus* is seen in the prosoma: opisthosoma length ratio. It equals 0.54–0.57 in the new species vs. only 0.17–0.19 in our well-fixed specimens of *U. elongatus* and 0.21 based on our measurements of the original line drawing of the type-specimen. Furthermore, 28S sequences are 0.9% different and *cox*1 sequences are 13.3% different between the 2 species.

Uvulifer pequenae can be further distinguished from *U. semicircumcisus* by somewhat smaller eggs (71–81 in the new species vs. 80–102 in *U. semicircumcisus*). The prosoma: opisthosoma length ratio in *U. pequenae* is also larger compared to *U. semicircumcisus* (0.54–0.57 in the new species vs. 0.28–0.41 in *U. semicircumcisus*). Additionally, *U. semicircumcisus* has only been reported from North America, whereas this new species is from the Peruvian Amazon.

Uvulifer pequenae can be further distinguished from *U. spinatus* by a larger ventral sucker (39–40 x 45–48 in the new species vs. 21–28 x 28–35 in *U. spinatus*). The prosoma: opisthosoma length ratio in *U. pequenae* is also larger compared to *U. spinatus* (0.54–0.57 in the new species vs. 0.28–0.41 in *U. spinatus*). Our sequence of *U. pequenae* 28S was similar to *U. spinatus*; the 2 species differ by 0.4% which is similar or greater than the differences recorded between other congeneric species within the Diplostomoidea Poirier, 1886 (Locke et al., 2018;

Achatz et al., 2019d). For example, 28S sequences of 3 species of *Parastrigea* Szidat, 1928 published by Hernández-Mena et al. (2017) differ by only 0.09–0.71% (1 to 8 bases different out of 1,132). The previously published *cox*1 sequences of *U. spinatus* were not homologous with the sequence obtained in our study.

Uvulifer pequenae can be further distinguished from *U. weberi* by a larger oral sucker (68–77 x 88–99 in the new species vs. 45–57 x 48–57in *U. weberi*) and larger holdfast organ (133–136 x 99–114 in the new species vs. 60–95 x 60–80 in *U. weberi*). The prosoma: opisthosoma length ratio in *U. pequenae* is larger compared to *U. weberi* (0.54–0.57 in the new species vs. 0.41–0.44 in *U. weberi* based on our specimens, and 0.35 based on the original line drawing of the type-specimen). The 28S sequence of *U. weberi* differs by 1.3% from that of *U. pequenae*, while *cox*1 sequences differ by 12.9%.

Uvulifer pequenae can be distinguished from *U. ambloplitis* as originally described by Hunter (1933) by having smaller eggs (71–81 long in the new species vs. 90–99 long in *U. ambloplitis*). The vitelline follicles do not reach the anterior margin of testes in *U. ambloplitis*, but extend anteriorly well beyond this level in the new species. Our sequences of *U. ambloplitis* and *U. pequenae* differ from each other by 1.4% in 28S and 12.9% in *cox*1. Additionally, adult *U. ambloplitis* have not been reported from outside the Nearctic.

Uvulifer pequenae is morphologically closest to *U. prosocotyle*, especially in the prosoma: opisthosoma length ratio (0.54–0.57 in the new species vs. 0.46–0.77 in our specimens of *U. prosocotyle* and 0.75 based on the original line drawing of the type-specimen). The 2 species differ in the egg size (71–81 long in the new species vs. 83–90 long in *U. prosocotyle*), and the relative extent of vitelline fields. The vitellarium-free zone occupies the first 13–16% of the opisthosoma in the new species compared to approximately 22–33% in our specimens of *U.*

prosocotyle. The vitellarium of *U. pequenae* extends to approximately halfway between the anterior margin of the ovary and the anterior margin of the opisthosoma. In contrast, the vitellarium of *U. prosocotyle* extends to approximately the anterior margin of the ovary. *Uvulifer prosocotyle* also has a very distinctive 'neck' region that is much narrower than the rest of the opisthosoma, whereas *U. pequenae* does not have this narrow part of the opisthosoma. Specimens of both *U. pequenae* and *U. prosocotyle* used in our study were heat-killed and fixed in the same manner. While morphology of both species is very similar, the sequence divergence is very substantial at 1.4% in 28S sequence and 12.9% in *cox*1. Complete comparison of metric characters for *U. pequenae* and *U. prosocotyle* is provided in Table 7.

Uvulifer batesi n. sp.

(Fig. 10)

Description [Based on 2 fully mature specimens]: Body 1,291–1,319 long, comprising prosoma and opisthosoma; prosoma oval, ventrally concave, 307–335 long, with maximum width at midway (251–285); opisthosoma elongated, 1,032–1,034, gradually widening toward bell-shaped posterior end (170–195). Prosoma: opisthosoma length ratio 0.31–0.33. Prosoma devoid of tegumental spines, opisthosoma (excluding bell-shaped posterior end) covered by tegumental spines. Oral sucker nearly terminal, 43–44 x 48–51. Prepharynx absent. Pharynx oval, overlapping with oral sucker, 23–25 x 20. Esophagus about equal in length with pharynx. Cecal bifurcation in anterior third of prosoma. Ceca slender, blind, extending to near posterior end of opisthosoma. Ventral sucker delicate, much smaller than oral sucker, 25–26 x 29–31, located 37–39% of the prosoma length from the anterior end. Holdfast organ 105 x 85, located immediately posterior to ventral sucker (46–47% of the prosoma length from the anterior end),

Table 7. Metric characters of new *Uvulifer* spp. from Peru and the most morphologically similar congeners from the New World. Measurements of *Uvulifer spinatus* taken from López-Jiménez et al. (2018). Range values are followed by mean after semicolon.

Species	*Uvulifer pequenae* n. sp. (n = 2)	*Uvulifer batesi* n. sp. (n = 2)	*Uvulifer prosocotyle* (n = 4)	*Uvulifer spinatus* (n = 13)
Geographic origin of material	Peru	Peru	Brazil	Mexico
Overall body length	1,403–1,432; 1,418	1,291–1,319; 1,305	1,060–1,439; 1,285	1,161–1,782; 1,499
Prosoma length	480–517; 499	307–335; 321	436–496; 460	276–439
Prosoma width	304–318; 311	251–285; 268	221–257; 235	204–227
Opisthosoma length	922–932; 927	1,032–1,034; 1,033	644–983; 846	800–1,327
Opisthosoma width	202–236; 219	170–195; 183	168–203; 183	110–195
Oral sucker length	68–77; 73	43–44; 44	55–73; 63	57–71; 61
Oral sucker width	88–99; 94	48–51; 50	106–113; 109	53–74; 62
Pharynx length	45–56; 51	23–25; 24	48–58; 54	34–46; 37
Pharynx width	34–37; 36	20	38–51; 43	29–35; 32
Ventral sucker length	39–40; 40	25–26; 26	35–38; 37	21–28; 24
Ventral sucker width	45–48; 47	29–31; 30	42–47; 45	28–35; 31
Holdfast organ length	133–136; 135	105	73–106; 88	88–121; 97
Holdfast organ width	99–114; 107	85	68–80; 75	97–125; 108
Ovary length	79–85; 82	Obscured by uterus	56–70; 62	49–72; 59
Ovary width	82–91; 87	Obscured by uterus	60–74; 65	56–64; 60
Anterior testis length	167–173; 170	91–94; 93	118–150; 136	80–144; 113
Anterior testis width	142–156; 149	85–97; 91	122–146; 131	91–125; 108
Posterior testis length	97–153; 125	97–107; 102	119–171; 138	78–139; 104
Posterior testis width	77–82; 80	94–97; 96	116–137; 124	89–124; 107
Genital cone length	60–65; 63	74–80; 77	61–94; 78	71–117; 89
Genital cone width	94–97; 96	80–86; 83	55–88; 67	–
Ejaculatory pouch length	142–156; 149	111	Not well observed	110–217; 172
Ejaculatory pouch width	71–85; 78	60–63; 62	Not well observed	64–109; 80
Egg number	2–5; 4	4–6; 5	0–3	–
Egg length	71–81; 76	76–87; 82	83–90; 88	65–81; 73
Egg width	46–57; 53	41–52; 47	43–44; 44	42–48; 44
Ventrolateral preputial lobe length	45–65; 55	68–99; 84	42–59; 50	–
Ventrolateral preputial lobe width	82–94; 88	130–142; 136	76–103; 89	–
Prosoma: opisthosoma length ratio	0.54–0.57; 0.56	0.31–0.33; 0.32	0.46–0.77; 0.56	0.28–0.41*
Oral sucker: ventral sucker width ratio	1.76–2.31; 2.04	1.39–1.52; 1.46	2.28–2.52; 2.44	1.67–2.33; 1.99
Anterior vitellarium-free zone: opisthosoma length	0.13–0.16; 0.15	0.25–0.28; 0.27	0.22–0.33; 0.25	–
Posterior vitellarium-free zone: opisthosoma length	0.11–0.12; 0.12	0.15–0.16; 0.16	0.12–0.14; 0.14	–
Anterior margin of ventral sucker positioned at	60–62% of prosoma length; 61%	37–39% of prosoma length; 38%	57–62% of prosoma length; 59%	–
Anterior margin of holdfast positioned at	72% of prosoma length	46–47% of prosoma length; 46.5%	66–72% of prosoma length; 69%	–
Anterior margin of ovary positioned at	32% of opisthosoma length	50–53% of opisthosoma length; 51.5%	27–45% of opisthosoma length; 36%	–

*Originally given as opisthosoma: Prosoma length ratio by López-Jiménez et al. (2018).

oval with ventral muscular portion having a deep, longitudinal slit-like opening and basal glandular portion embedded in the prosoma. Testes tandem, with smooth margins, anterior testis 91–94 x 85–97, posterior testis 97–107 x 94–97. Seminal vesicle subglobular, ventral to posterior testis, connected to ejaculatory duct; proximal ejaculatory duct funnel-like with proximal end wide and distal end narrowing and running antero-dorsally, then bending and running posteriorly; distal portion opening into a muscular ejaculatory pouch; ejaculatory pouch 111 x 60–63, draining posteriorly through narrow short male duct. Ovary appearing subspherical with smooth margin (but largely obscured by uterus in both specimens), immediately pretesticular (50–53% of the opisthosoma length from the anterior end). Ootype surrounded by Mehlis' gland, submedian (slightly dextral), intertesticular. Seminal receptacle not observed. Uterus ventral in opisthosoma, extending from a level slightly pre-ovarian to posterior margin of posterior testis, containing 4–6 eggs (76–87 x 41–52); distal uterus uniting with male duct and forming hermaphroditic canal; hermaphroditic canal descending into genital cone. Genital cone 74–80 x 80–86, extending into a highly bulbous copulatory bursa; copulatory bursa with prominent muscular ventrolateral preputial fold. Ventrolateral preputial fold 68–99 x 130–142. Vitelline follicles in opisthosoma, ventral, absent in the anterior 25–28% of the opisthosoma and posterior 15–16% of opisthosoma. Vitelline reservoir intertesticular, sinistral to ootype. Excretory vesicle I-shaped, with main stem dorsal in opisthosoma; main stem appearing wavy, ascending into prosoma and surrounding holdfast organ and giving rise to 6 secondary longitudinal branches that extend toward oral sucker; branches surrounding suckers and interconnected by network of anastomosing channels throughout prosoma. Excretory pore not observed.

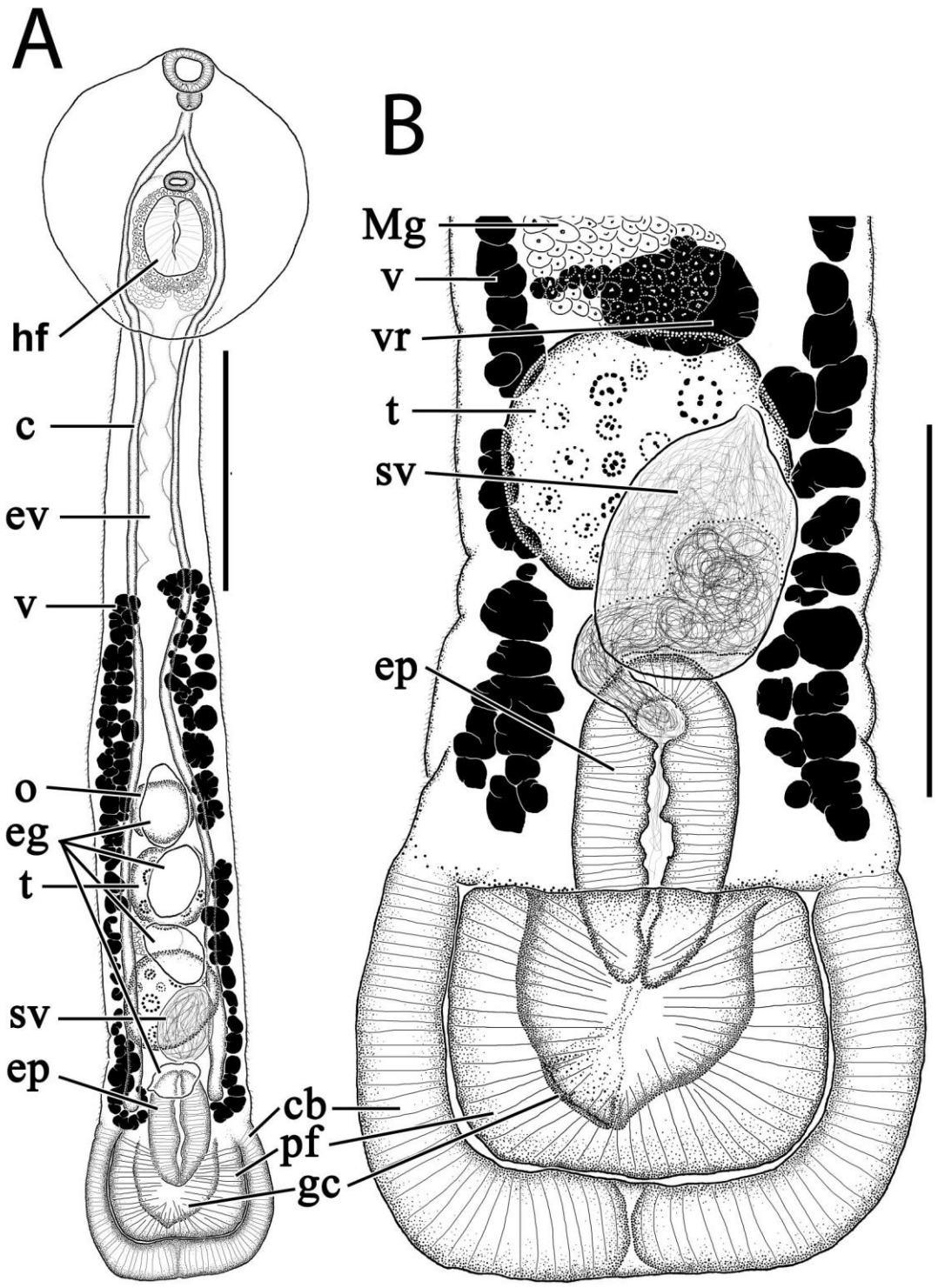

Figure 10. *Uvulifer batesi* n. sp. **(A**) Ventral view of holotype. (**B**) Ventral view of posterior body end of holotype with uterus omitted. Scale bars: A, 250 µm; B, 150 µm. Abbreviations: c, ceca; cb, copulatory bursa; eg, egg; ep, ejaculatory pouch; ev, excretory vesicle; gc, genital cone; hf, holdfast organ; Mg, Mehlis'gland; o, ovary; pf, preputial fold; sv, seminal vesicle; t, testis; v, vitelline follicle; vr, vitelline reservoir. (after Achatz et al., 2019a)

Taxonomic summary

Type host: Chloroceryle inda (Linnaeus) (Coraciiformes: Alcedinidae).

Site of infection: Small intestine.

Type locality: San Martín, Tocache Province, Cordillera Azul National Park, Río Pescadero, NE of Shapaja (8°10.694'S, 76°13.422'W), Peru, elev. 953 m above sea level.

Type specimens deposited: The type series consists of 2 fully mature specimens deposited in the Harold W. Manter Laboratory. Holotype: HWML 139983, labeled ex. *C. inda*, small intestine, Cordillera Azul National Park, Peru, 13 Nov 2013, coll. K. Patitucci; paratype: HWML-139984, labeled identical to the holotype. Symbiotype deposited in the Field Museum, Chicago (FMNH 3859910).

Representative DNA sequences: GenBank MK874321 (28S), MK871330 (*cox*1).

ZooBank registration: urn:lsid:zoobank.org:act:F23BE7CF-0942-404F-AD5F-E2E2D373A4AE

Etymology: The new species is named after Dr. John Bates in recognition of his contributions to the knowledge of South American birds and as the leader of the field crew that collected the new species.

Remarks

The new species clearly belongs to *Uvulifer* based on the combination of characteristic features such as the presence of a muscular ejaculatory pouch and a muscular copulatory bursa containing a retractile or protrusible genital cone partially surrounded by a ventrolateral preputial muscular fold.

Uvulifer batesi is easily distinguished from the New World congeners by the wide, bell-shaped copulatory bursa region at the posterior body end. This is the widest portion of the

opisthosoma in *U. batesi*, whereas the widest part of the opisthosoma in other New World congeners is at the testicular level.

Uvulifer batesi can also be distinguished from *U. elongatus*, *U. semicircumcisus*, *U. spinatus* and *U. weberi* by relatively shorter vitellarium. The vitellarium in all these 4 species occupies almost the whole length of the opisthosoma, whereas in *U. batesi* it is absent in the first 25–28% of the opisthosoma.

Uvulifer batesi can be further differentiated from *U. elongatus* by shorter body length (1,291–1,319 in the new species vs. 2,200–3,300 in *U. elongatus*), a much smaller ventral sucker (25–26 x 29–31 in the new species vs. 85–100 x100–120 in *U. elongatus*) and pharynx (23–25 x 20 in the new species vs. 45–55 x 30–37 in *U. elongatus*). In addition, *U. batesi* and *U. elongatus* differ by 0.9% in 28S sequences and 12.9% in *cox*1 sequences.

Uvulifer batesi can be further distinguished from *U. semicircumcisus* by a thinner opisthosoma (170–195 in the new species vs. 270–400 in *U. semicircumcisus*) and smaller ventral sucker (25–26 x 29–31 in the new species vs. 40–49 in diameter in *U. semicircumcisus*). Additionally, *U. semicircumcisus* has only been reported in North America, whereas *U. batesi* was found in Peruvian Amazon.

Uvulifer batesi can be further differentiated from the morphologically similar *U. spinatus* by the distribution of tegumental spines. In *U. batesi* the tegumental spines cover the majority of the opisthosoma, whereas in *U. spinatus* they only extend from the anterior margin of the opisthosoma to the anterior testis. Additionally, the 2 species can be differentiated by the more posteriorly positioned gonads in *U. batesi*, a smaller pharynx (23–25 x 20 in this new species vs. 34–46 x 29–35 in *U. spinatus*) and a smaller oral sucker: ventral sucker width ratio (1.39–1.52 in this new species vs. 1.67–2.33 in *U. spinatus*). The 28S sequence of *U. batesi* was similar to that

of *U. spinatus*; the 2 species differ by only 0.3%. The available *cox*1 sequences of *U. spinatus* were not homologous with our sequences. Complete comparison of metric characters for *U. batesi* and *U. spinatus* is provided in Table 7.

Uvulifer batesi can be further distinguished from *U. weberi* by the somewhat, relatively more posterior gonads in *U. batesi*. In addition, both 28S (1.3%) and *cox*1 (13.7%) sequences are quite different between the 2 species.

Uvulifer batesi can be further distinguished from *U. ambloplitis*, as originally described by Hunter (1933), by having a smaller oral sucker (43–44 x 48–51 in the new species vs. 94–120 diameter in *U. ambloplitis*), smaller pharynx (23–25 x 20 in our new species vs. 52–63 x 40–45 in *U. ambloplitis*), smaller ventral sucker (25–26 x 29–31 in our new species vs. 44–52 x 45–56 in *U. ambloplitis*), smaller eggs (76–87 in our new species vs. 90–99 in *U. ambloplitis*) and relatively longer fields of vitelline follicles that do not reach the anterior margin of testes in *U. ambloplitis*, but extend well beyond this level anteriorly in *U. batesi*. Our sequences of *U. ambloplitis* and *U. batesi* are 1.4% different in 28S and 15.1% different in *cox*1. As stated above, adult specimens of *U. ambloplitis* have only been reported in the Nearctic, whereas *U. batesi* is from the Peruvian Amazon.

Uvulifer batesi can be further differentiated from *U. prosocotyle* by the lower prosoma: opisthosoma length ratio (0.31–0.33 in the new species vs. 0.46–0.77 in our specimens of *U. prosocotyle* and 0.75 based off the original line drawing of the type-specimen). In addition, *U. prosocotyle* also has a very distinctive ‘neck’ region that is much narrower than the rest of the opisthosoma while *U. batesi* does not have this narrowed part of the opisthosoma. In addition, the 2 species differ by 1.4% in 28S sequences and by 13.1% in *cox*1 sequences.

Uvulifer batesi can be further distinguished from *U. pequenae* by the lower prosoma: opisthosoma length ratio (0.31–0.33 in the new species vs. 0.54–0.57 in *U. pequenae*) and the distribution of tegumental spines. The tegumental spines of *U. batesi* cover most of the opisthosoma, but are completely absent on the prosoma. In contrast, the anterior 25% of the opisthosoma and entire prosoma have tegumental spines in *U. pequenae*. The sequences of 28S were very close with only 0.2% difference; however, the *cox*1 sequences showed a much greater difference of 10%.

Molecular phylogenies

Upon trimming to the length of the shortest sequence the 28S alignment was 1,133 bp long. The phylogenetic tree resulting from the BI analysis contained 6 *Uvulifer* clades (Fig. 11). The clade 1 (88%) included recently published *Uvulifer* sp. (GenBank MK604825) from South Africa and a well-supported clade (99%) of *U. ambloplitis* + *U. prosocotyle* + *U. weberi* + *Uvulifer* sp. (GenBank MF568569). Notably, this clade included species from the Afrotropics, Nearctic, and Neotropics. The clade 2 (97%) included both of our isolates of *U. elongatus* collected from the Amazonas (Lábrea) and Mato Grosso (Pantanal) states in Brazil. The clade 3 (97% support) was composed of *Uvulifer* sp. (GenBank MF568575) + a well-supported clade (100%) of *Uvulifer* sp. (GenBank MF568574) + *Uvulifer* sp. (GenBank MK874327). This clade was composed of only metacercariae from species from the Nearctic and Neotropics. The clade 4 (94% support) included *U. spinatus* + *Uvulifer* sp. (GenBank MF398332). The clades 5 and 6 included a single species each, *U. pequenae* and *U. batesi*.

Upon trimming to the length of the shortest sequence the *cox*1 alignment was 451 bp long. While the branch topology of the *Uvulifer* tree was reasonably resolved, the support of the

majority of nodes was rather weak (Fig. 12). The 2 new species from Peru appeared on the tree as sister taxa to the rest of the species in the genus. Despite some difference in the composition of the included species the 28S and *cox*1 phylogenies had an overall very similar branch topology.

Genetic variation

The interspecific divergence in 28S sequences of *Uvulifer* spp. was generally low (0.1–2.2% or 1–25 bases out of 1,132). In contrast, *cox*1 sequences had much greater interspecific variation (9.3–15.3% or 42–69 bases out of 451). Although the 2 new *Uvulifer* species from the Peruvian Amazon were very similar in 28S sequences (0.2% or 2 bases out of 1,132), they were 10% different (45 bases out of 451 bases) in *cox*1.

Uvulifer pequenae and its morphologically closest congener *U. prosocotyle* differ by 1.4% (16 bases out of 1,132 bases) in 28S sequences and 12.9% (58 bases out of 451 bases) in *cox*1. *Uvulifer batesi* and its morphologically closest congener *U. spinatus* differ by 0.3% (3 bases out of 1,132 bases) in 28S sequences (compatible *cox*1 sequences of *U. spinatus* are not available). Pairwise nucleotide comparisons among all *Uvulifer* spp. are provided in Tables 8 and 9. It is noteworthy that our isolate of *U. elongatus* from Pantanal, Brazil had a single mixed base (a double peak) in its 28S sequence, while our isolate of *U. elongatus* from Lábrea, Brazil did not have any mixed bases in 28S. There was only 0.5% difference (2 out of 426 bases) in their *cox*1 sequences.

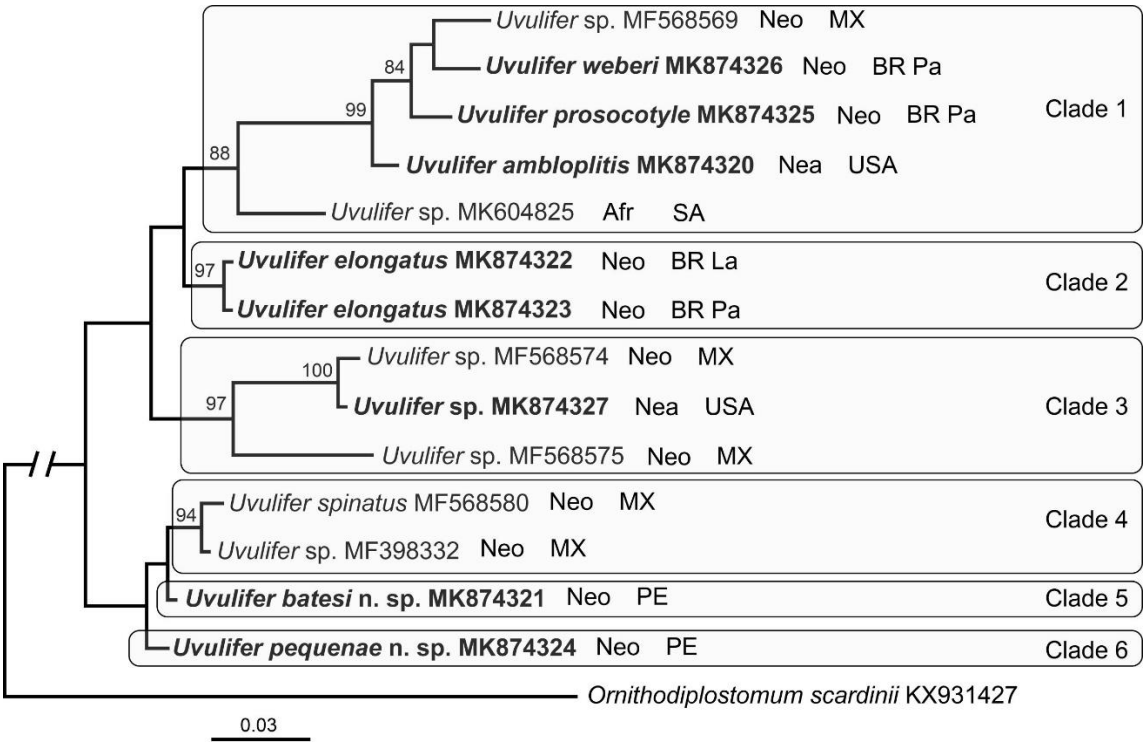

Figure 11. Phylogenetic interrelationships among 14 *Uvulifer* taxa based on Bayesian Inference (BI) analysis of partial 28S rRNA gene sequences. Bayesian Inference posterior probability values lower than 70% (BI) are not shown. New sequences obtained in this study are in bold. Branch length scale bar indicates number of substitutions per site. GenBank accession numbers and the biogeographical realm and geographic origin are provided after the names of species. Abbreviations for biogeographical realms: Afr, Afrotropical realm; Nea, Nearctic realm; Neo, Neotropical realm. Abbreviations for geographic origin: BR La, Lábrea site in Brazil; BR Pa, Pantanal site in Brazil; MX, Mexico; PE, Peru; SA, South Africa; USA, United States of America. (after Achatz et al., 2019a)

Discussion

The 2 new species of *Uvulifer* described herein represent the first species of *Uvulifer* described from Peru, and the 7th and 8th species of *Uvulifer* species in the New World. Our study is the first to provide DNA sequence data from *U. ambloplitis*, *U. elongatus*, *U. prosocotyle* and *U. weberi*. Although there have been a number of studies involving *Uvulifer* (e.g., Boyd & Fry, 1971; Muzzall et al., 2011; Flores-Lopes, 2014), our study is only the fourth molecular phylogenetic study to produce DNA sequence data sourced from adult *Uvulifer* spp. (Hernández-Mena et al., 2017; López-Jiménez et al. 2018; Hoogendoorn et al., 2019), and only the second study to produce DNA sequence data from named adult material (López-Jiménez et al., 2018).

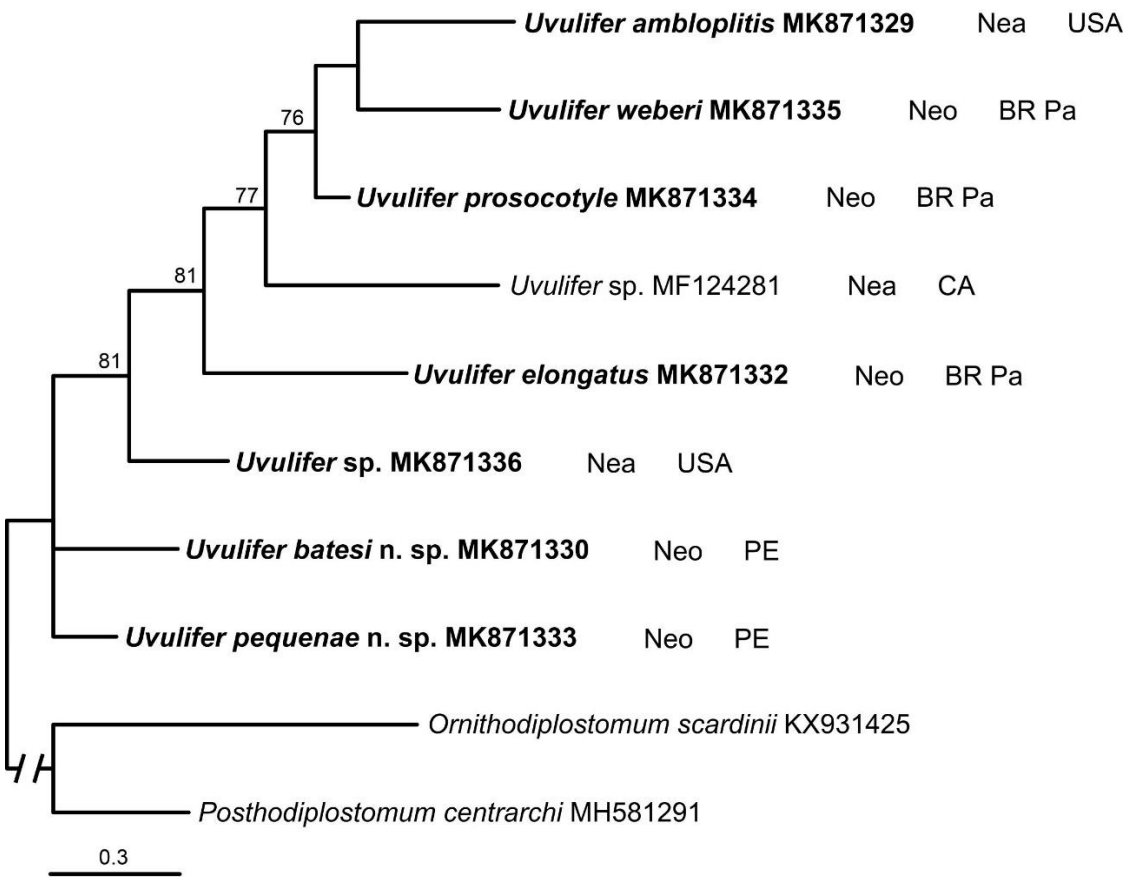

Figure 12. Phylogenetic interrelationships among 8 *Uvulifer* taxa based on Bayesian Inference (BI) analysis of partial *cox*1 mtDNA sequences. Bayesian Inference posterior probability values lower than 70% (BI) are not shown. New sequences obtained in this study are in bold. Branch length scale bar indicates number of substitutions per site. GenBank accession numbers, the biogeographical realm and the geographic origin are provided after the names of species. Abbreviations for biogeographical realms: Nea, Nearctic realm; Neo, Neotropical realm. Abbreviations for geographic origin: BR Pa, Pantanal site in Brazil; CA, Canada; PE, Peru; USA, United States of America. (after Achatz et al., 2019a)

The interspecific genetic variation among partial 28S sequences was lower than demonstrated by López-Jiménez et al. (2018) for *U. spinatus* and other unnamed lineages of *Uvulifer*. Our 28S sequences of *Uvulifer* from South and North America demonstrated 0.2–1.6% interspecific divergence levels (Table 8), which is lower than the range of 1.3–1.6% for interspecific differences reported by López-Jiménez et al. (2018). Interspecific divergence in our partial *cox*1 sequences showed levels of differences similar to those reported by López-Jiménez et al. (2018). Newly generated *cox*1 sequences showed 9.3–15.1% difference among species

(Table 9), whereas López-Jiménez et al. (2018) reported 9.3–12.5% differences. The 2 genetically closest named species of *Uvulifer* in our dataset (*U. batesi* and *U. pequenae*), had only 2 nucleotide difference in 28S while demonstrating a much greater 10% difference in *cox*1 sequences. This suggests that as few as 2 bases difference (assuming high sequence quality) in 28S may be sufficient to differentiate between species in this genus, although it cannot be excluded that some species may have identical 28S sequences.

Our newly generated *cox*1 sequences cover the same region of *cox*1 as the vast majority of published *cox*1 sequences of diplostomoideans (e.g., sequences originating from Blasco-Costa & Locke, 2017; Hernández-Mena et al., 2017; Hoogendoorn et al., 2019). López-Jiménez et al. (2018) opted to amplify and sequence a different region of *cox*1 for their *Uvulifer* spp. We attempted amplification of the region sequenced by López-Jiménez et al. (2018) from our 2 new species. The PCRs were unsuccessful, although we did not experience problems amplifying and sequencing the 28S fragment and the standard "barcoding" region of the *cox*1 gene. Only 2 of the newly generated *cox*1 sequences (from metacercaria GenBank MK871336 and *U. prosocotyle* Genbank MK871334) overlapped with the region of the *cox*1 gene sequenced by López-Jiménez et al. (2018). Their sequence MF568574 and our metacercaria from Minnesota differ in 28S only by a single nucleotide; however, in *cox*1 they differ by 4.9% (14 bases out of 283). This level of divergence is much lower than differences seen between other named *Uvulifer* species in the same region of *cox*1 (usually ~10% difference or more). It should be noted that according to López-Jiménez et al. (2018) the *cox*1intraspecific variation in their material did not exceed 1.8%. Sequencing and morphological examination of a greater diversity of adult specimens from broader geographic area is necessary to determine if the metacercaria

Table 8. Pairwise comparisons of partial sequences of the 28S rRNA gene between *Uvulifer* species included in this study. Percentage differences are given above diagonal and the number of variable nucleotide positions is given below the diagonal. The 28S results are based on a 1,132 bp long alignment.

	1. MK874320	**2.** MK874321	**3.** MK874323	**4.** MK874324	**5.** MK874325	**6.** MK874326	**7.** MF568582	**8.** MK874327	**9.** MF398332	**10.** MF568569	**11.** MF568674	**12.** MF568575	**13.** MK604825
1. *Uvulifer ambloplitis* MK874320	–	1.4%	1.2%	1.4%	0.5%	0.5%	1.5%	1.6%	1.4%	0.7%	1.7%	2%	1.2%
2. *Uvulifer batesi* n. sp. MK874321	16	–	0.9%	0.2%	1.4%	1.3%	0.3%	1.4%	0.2%	1.5%	1.3%	1.7%	1.2%
3. *Uvulifer elongatus* MK874323	13	10	–	0.9%	1.5%	1.4%	1.2%	1.2%	1.1%	1.6%	1.3%	1.5%	0.8%
4. *Uvulifer pequenae* n. sp. MK874324	16	2	10	–	1.4%	1.3%	0.4%	1.4%	0.4%	1.5%	1.3%	1.7%	1.2%
5. *Uvulifer prosocotyle* MK874325	6	16	17	16	–	0.5%	1.5%	1.6%	1.4%	0.5%	1.7%	2%	1.2%
6. *Uvulifer weberi* MK874326	6	15	16	15	6	–	1.6%	1.7%	1.5%	0.5%	1.8%	2%	1.6%
7. *Uvulifer spinatus* MF568582	17	3	13	5	17	18	–	1.7%	0.1%	1.6%	1.6%	2%	1.3%
8. *Uvulifer* sp. MK874327	18	16	14	16	18	19	19	–	1.6%	1.7%	0.1%	1.3%	1.4%
9. *Uvulifer* sp. MF398332	16	2	12	4	16	17	1	18	–	1.5%	1.5%	1.9%	1.2%
10. *Uvulifer* sp. MF568569	8	17	18	17	6	6	18	19	17	–	1.8%	2.2%	1.6%
11. *Uvulifer* sp. MF568674	19	15	15	15	19	20	18	1	17	20	–	1.4%	1.5%
12. *Uvulifer* sp. MF568575	22	19	17	19	22	23	22	15	21	25	16	–	1.6%
13. *Uvulifer* sp. MK604825	14	14	9	14	14	18	15	16	14	18	17	18	–

Table 9. Pairwise comparisons of partial sequences of the *cox*1 mtDNA gene between *Uvulifer* species included in this study. Percentage differences are given above diagonal and the number of variable nucleotide positions is given below the diagonal. Results are based on a 451 bp long alignment.

	1. MK871329	**2.** MK871330	**3.** MK871332	**4.** MK871333	**5.** MK871334	**6.** MK871335	**7.** MK871336	**8.** MF124281
1. *Uvulifer ambloplitis* MK871329	–	15.1%	14.6%	12.9%	10.4%	11.5%	13.5%	13.7%
2. *Uvulifer batesi* n. sp. MK871330	68	–	12.9%	10%	13.1%	13.7%	11.3%	15.3%
3. *Uvulifer elongatus* MK871332	66	58	–	13.3%	11.3%	13.5%	14.2%	14.4%
4. *Uvulifer pequenae* n. sp. MK871333	58	45	60	–	12.9%	12.9%	10.4%	14.2%
5. *Uvulifer prosocotyle* MK871334	47	59	51	58	–	9.3%	10.4%	11.3%
6. *Uvulifer weberi* MK871335	52	62	61	58	42	–	13.3%	13.3%
7. *Uvulifer* sp. MK871336	61	51	64	47	47	60	–	12%
8. *Uvulifer* sp. MF124281	62	69	65	64	51	60	54	–

from our material is an independent species or represents a genetically divergent population of a known species.

Six species of kingfishers occur in the Americas. *Megaceryle alcyon* inhabits widespread areas of North America north of Mexico and may also winter in Central and South America. *Megaceryle torquata* inhabits ranges from the Rio Grande valley of North America south throughout Central America and South America. *Chloroceryle americana* is distributed throughout the southwestern United States south to central Argentina. *Chloroceryle amazona* ranges from Central America south to northern Argentina; the American pygmy kingfisher, *Chloroceryle aenea* (Pallas), ranges from southern Mexico south throughout central South America. *Chloroceryle inda* range extends from Nicaragua to Paraguay (Remsen, 1991). Our phylogenetic analyses included *Uvulifer* spp. from 4 New World kingfisher species: *M. alcyon, M. torquata, C. americana* and *C. inda*. In the phylogeny resulting from our analysis of 28S (Fig. 11), neither of the well-supported clades that included more than 1 species of *Uvulifer* was limited to a single kingfisher species. In part, this may be the result of the strong overlap of distributions of the South American kingfisher species. It is known that a species of kingfisher can be host to multiple species of *Uvulifer*, for instance, *U. pequenae* and *U. batesi* both parasitize *C. inda* and at least 3 species of *Uvulifer* parasitize *M. alcyon* (Hernández-Mena et al., 2017; López-Jiménez et al., 2018; present data). However, the potential for a single *Uvulifer* species to infect multiple species of kingfisher has not been previously tested using molecular tools.

The phylogenetic tree based on the 28S alignment (Fig. 11) revealed 2 strongly supported clades of *Uvulifer* containing specimens from distant geographical locations. Clade 1 included *Uvulifer* sp. from Afrotropical realm, *U. ambloplitis* from Nearctic and *Uvulifer* sp., *U. weberi*,

and *U. prosocotyle* from Neotropics. The clade 3 included 2 unidentified species-level lineages distributed in Mexico and Central America (López-Jiménez et al., 2018) and a form from the northern U.S.A. This likely indicates at least 2 independent dispersal events in the evolutionary history of the New World *Uvulifer*. The interrelationships and phylogeographic history of *Uvulifer* will likely be better resolved once DNA sequence data are available from a greater diversity of *Uvulifer* species including those from the Eastern Hemisphere.

The branch topology in the *cox*1 phylogenetic tree was not fully resolved and had overall lowed support values likely due to the mutation saturation effect. Somewhat higher branch support values in the *cox*1 tree within *Uvulifer* reported by López-Jiménez et al. (2018) are likely explained by the fact that these authors sequenced a different, somewhat shorter and less variable region of *cox*1 gene. Our results indicate that while *cox*1 sequences are a great tool for species differentiation, they should be used with caution for phylogenetic inference at higher taxonomic levels.

The result of our *cox*1 phylogeny (Fig. 12) confirmed the low utility of *cox*1 sequence data for phylogenetic inference in this digenean group that was suggested in the recent major publications on this group and digeneans overall (Locke et al., 2018; Pérez-Ponce de León & Hernández-Mena, 2019). Regardless, utilization of ribosomal as well as mitochondrial sequence data as tool for assisting with differentiating among species greatly enhances the power of taxonomic investigations within the Diplostomidae.

Our specimens of *U. ambloplitis* closely conform morphologically to the form originally described as *Uvulifer claviformis* Dubois et Rausch, 1948. Boyd & Fry (1971) later noted that Dubois viewed *U. claviformis* as a synonym of *U. ambloplitis* based on materials from Boyd & Fry (1971) and other materials in a personal communication. We believe the differences between

the 2 forms can be possibly explained by the varying levels of contraction after fixation and/or levels of maturity as noted by Boyd & Fry (1971). Specimens morphologically identical to *U. ambloplitis* as described by Hunter (1933) should be sequenced for an adequate molecular and morphological comparison and a taxonomic conclusion regarding the form described by Dubois & Rausch (1948) and other previously synonymized species.

The overwhelming majority of ecological studies that report *Uvulifer* spp. did not include DNA sequence data (e. g., Boyd & Fry, 1971; Pérez-Ponce de León et al., 2010; Muzzall et al., 2011; McAllister et al., 2013; Flores-Lopes, 2014, Zimmermann et al., 2016; Hollander et al., 2019). Based on our results, it is clear that the diversity of *Uvulifer* in the New World is greater than previously recognized. At present, only 2 named species are currently known in North America north of Mexico (Boyd & Fry, 1971; López-Jiménez et al., 2018). Likely, many of the previous ecological studies dealing with larval stages of *Uvulifer* included more than a single *Uvulifer* species. Detailed molecular and morphological comparisons should provide a solution for this problem.

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Associated publication

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CHAPTER VI

PHYLOGENETIC RELATIONSHIPS, EXPANDED DIVERSITY AND DISTRIBUTION OF CRASSIPHIALA (DIGENEA, DIPLOSTOMIDAE), AN AGENT OF 'BLACK SPOT' DISEASE IN FISH

Introduction

Crassiphiala Van Haitsma, 1925 (Diplostomidae: Crassiphialinae) is a monotypic genus of diplostomid digeneans parasitic in kingfishers (Alecedinidae Rafinesque) (Dubois, 1968). The type-species *Crassiphiala bulboglossa* Van Haitsma, 1925 was described from the intestine of the belted kingfisher *Megaceryle alcyon* (Linnaeus) from Michigan, USA (Van Haitsma, 1925) and since then reported only in the Nearctic (Preble & Harwood, 1944; Dubois & Rausch, 1948; Hoffman, 1956; Dubois, 1969; Boyd & Fry, 1971; Scott, 1984; Niewiadomska, 2002d; Muzzall et al., 2011). The life cycle of *C. bulboglossa* is similar to members of the genus *Uvulifer* Yamaguti, 1934 and includes planorbid snails and fishes as intermediate hosts (Hoffman, 1956). Notably, *C. bulboglossa* has a *Neascus*-type metacercaria that normally encysts in fish skin and is often melanized by the fish host. This infection is often referred to as the 'black spot' disease (Hunter, 1933; Hoffman, 1956; Niewiadomska, 2002d; McAllister et al., 2013). Adult *Crassiphiala* are characterized, among other features, by a large holdfast organ, rudimentary or absent ventral sucker and the absence of an ejaculatory pouch (Niewiadomska, 2002d).

Crassiphiala is the type-genus of the subfamily Crassiphialinae Sudarikov, 1960. The most recent revision of the Crassiphialinae by Niewiadomska (2002d) included 15 genera; however, adults of only 4 of these genera have been included in prior molecular phylogenetic

analyses based on the 28S rRNA gene. The molecular phylogenetic studies that included more than 3 genera of the Crassiphialinae have shown mixed support for the monophyly of the subfamily (see Blasco-Costa & Locke, 2017; Hernández-Mena et al., 2017; López-Jiménez et al., 2018; Achatz et al., 2019d). Despite very weak support or the lack of support evident from their phylogenetic trees, some authors have repeatedly suggested that this subfamily may warrant elevation to family (Blasco-Costa & Locke, 2017; Hernández-Mena et al., 2017; Locke et al., 2018). Moreover, no prior molecular phylogenetic study included representatives of *Crassiphiala*, the type genus of the Crassiphialinae. The purpose of this study is the demonstration of the phylogenetic placement of *Crassiphiala* using DNA sequence data for the first time as well as the presence of this genus in South America. Formal morphological and taxonomic descriptions of the genetic lineages presented herein will be published separately.

Materials & Methods

Specimens

We obtained adult specimens of *Crassiphiala* from intestines of *M. alcyon* collected in Clearwater (1 specimen) and St. Louis (1 specimen) Counties in Minnesota, U.S.A. (collecting permit MB072162-0) and a single ringed kingfisher *Megaceryle torquata* (Linnaeus) collected in Pantanal, Fazenda Retiro Novo, Municipality of Poconé, Mato Grosso State, Brazil (collecting permit 10698 approved by the Instituto Chico Mendes de Conservação da Biodiversidade), using corresponding federal and state collecting permits. In addition, metacercaria of *Crassiphiala* were collected from the skin and fins of yellow perch *Perca flavescens* Mitchill from Cass County, Minnesota, central mudminnow *Umbra limi* Kirtland from Hubbard County, Minnesota and *Chrosomus eos* Cope from St. Louis County, Minnesota. One sample was obtained from a

frozen carcass of *M. alcyon* that died after flying into a glass window. These specimens were directly fixed in 70% ethanol (Table 10).

Phylogenetic analyses

Phylogenetic relationships of *Crassiphiala* were analyzed using 28S and *cox*1 datasets as separate alignments. Three *cox*1sequences (2 from the lineage 2 and 1 from the lineage 4) were much shorter than the rest and therefore not included in the alignment, although they were submitted to the GenBank (Table 10). These shorter sequences were identical to their longer counterparts. Newly obtained and previously published sequences were aligned using ClustalW implemented in MEGA7 (Kumar et al., 2016); both alignments were trimmed to the length of the shortest sequence. The cyathocotylid *Suchocyathocotyle crocodili* (Yamaguti, 1954) (GenBank accession MK650450) was selected as outgroup in the 28S analysis based on the topology presented by Achatz et al. (2019d). *Uvulifer* sp. (GenBank accession MF124281; Blasco-Costa & Locke, 2017) was selected as outgroup in the *cox*1 analysis based on the results of our 28S analysis and genetic distances.

The 28S alignment included newly generated sequences of 6 taxa of *Crassiphiala* and previously published sequences of 18 members of the Diplostomidae Poirier, 1886, 1 member of the Proterodiplostomidae Dubois, 1936 and 12 members of the Strigeidae Railliet, 1919. The *cox*1alignment included newly generated sequences of 7 taxa of *Crassiphiala*. Phylogenetic analyses were conducted as described by Achatz et al. (2019d). The trees were visualized in FigTree ver. 1.4 software (Rambaut, 2016) and annotated in Adobe Illustrator®.

Table 10. List of *Crassiphiala* samples used in the phylogenetic analyses of 28S rRNA and *cox*1 mtDNA genes including sample size (n), Harold W. Manter Laboratory (HWML) voucher numbers, their host species, geographical origin of material and GenBank accession numbers.

Digenean taxa	Host species	Geographic origin	Museum No.	Accession numbers	
				28S	*cox*1
Crassiphiala lineage 1 (n=2)	*Megaceryle alcyon*	USA	HWML-216012	MN200252, MN200253	MN193951
Crassiphiala lineage 2 (n=1)	*M. alcyon*	USA	–	MN200254	MN193952
Crassiphiala lineage 2 (n=1)	*Chrosomus eos*	USA	–	MN200255	MN193953
Crassiphiala lineage 2 (n=2)	*Umbra limi*	USA	–	MN200256	MN193954, MN193955
Crassiphiala lineage 3 (n=1)	*Perca flavescens*	USA	–	MN200257	MN193956
Crassiphiala lineage 4 (n=3)	*Megaceryle torquata*	Brazil	HWML-216013	MN200258–MN200260	MN193957, MN193958
Crassiphiala lineage 5 (n=2)	*M. torquata*	Brazil	HWML-216014	MN200261	MN193959, MN193960

Results

Molecular phylogenies

Upon trimming to the length of the shortest sequence the 28S alignment was 1,137 bp long. Similar to the results of several previous studies (e.g. Blasco-Costa & Locke, 2017; Hernández-Mena et al., 2017; Achatz et al., 2019d) the phylogenetic tree resulting from the BI analysis demonstrated the non-monophyletic nature of the Diplostomidae and Strigeidae (Fig. 13). Members of the Diplostomidae formed 6 clades: 1) Crassiphialinae clade 1 which included *Crassiphiala* + *Uvulifer* (100% supported), 2) Crassiphialinae clade 2 which included *Bolbophorus* Dubois, 1935 + *Ornithodiplostomum* Dubois, 1936 + *Posthodiplostomum* Dubois, 1936 (88% supported), 3) *Hysteromorpha* Lutz, 1931, 4) *Austrodiplostomum* Szidat & Nani, 1951 + *Diplostomum* von Nordmann, 1832 + *Tylodelphys* Diesing, 1850 (100% supported), 5) *Neodiplostomum* Railliet, 1919 and 6) *Alaria* Schrank, 1788 (99% supported). All sequenced lineages of *Crassiphiala* formed a strongly supported clade (98% supported); *Crassiphiala* lineage 4 from Brazil formed a sister group to all other *Crassiphiala* isolates (Fig. 13). Members

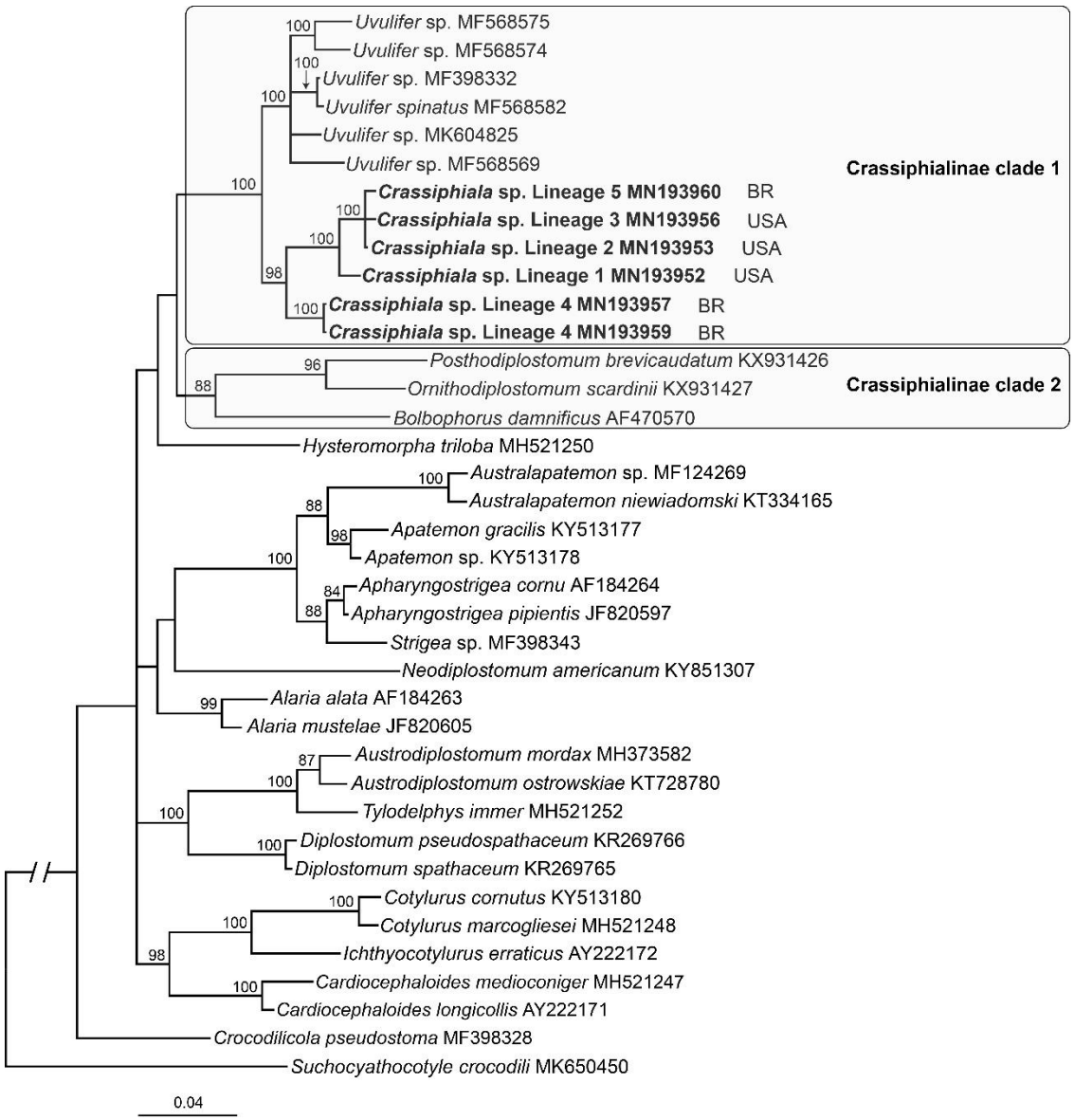

Figure 13. Phylogenetic position of *Crassiphiala* species within the Diplostomoidea based on Bayesian Inference (BI) analysis of partial 28S rRNA gene sequences. Members of the subfamily Crassiphialinae as currently recognized are indicated by the shaded rectangles. Bayesian Inference posterior probability values lower than 80% are not shown. New sequences obtained in this study are bolded. Scale bar indicates number of substitutions per site. Abbreviations: BR, Brazil; USA, United States of America. (after Achatz et al., 2019c)

of the Strigeidae formed 2 strongly supported clades. The first clade (100% supported) included *Apharyngostrigea* Ciurea, 1927 + *Strigea* Abildgaard, 1790 + *Apatemon* Szidat, 1928+ *Australapatemon* Sudarikov, 1959; the second clade (98% supported) included *Cardiocephaloides* Sudarikov, 1959 + *Cotylurus* Szidat, 1928 + *Ichthyocotylurus* Odening, 1969.

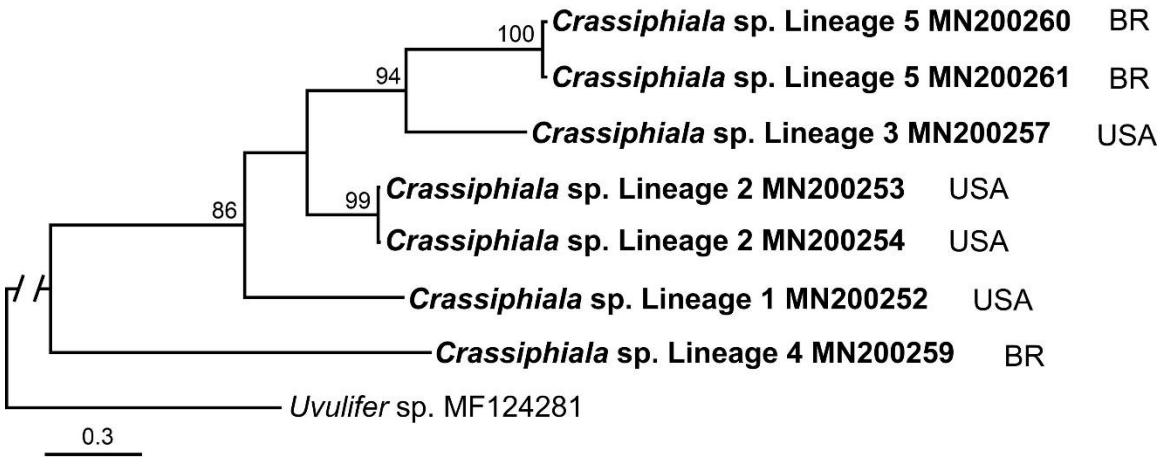

Figure 14. Phylogenetic interrelationships among *Crassiphiala* lineages based on Bayesian Inference (BI) analysis of partial *cox*1 mtDNA sequences. Bayesian Inference posterior probability values lower than 80% are not shown. All sequences of *Crassiphiala* included in our analysis are new and bolded. Scale bar indicates number of substitutions per site. Abbreviations: BR, Brazil; USA, United States of America. (after Achatz et al., 2019c)

The internal interrelationships among available isolates of *Crassiphiala* were studied using the 392 bp long *cox*1 alignment. While the overall topology in the *cox*1 tree was similar to that of the *Crassiphiala* clade in the 28S tree, the much more variable *cox*1 sequences provided added resolution in form of the well-supported cluster of *Crassiphiala* lineages 3 and 5 which was unresolved in the 28S tree. *Crassiphiala* lineage 4 formed a sister group with all other *Crassiphiala* isolates, although with a somewhat lower support than in the 28S gene tree (Fig. 14).

Genetic variation

Pairwise nucleotide comparisons of 28S sequences among all unique *Crassiphiala* isolates are provided in Table 11. The divergence in 28S sequences of *Crassiphiala* lineages was generally low (0.2–2.4%). One of the 28S sequences of *Crassiphiala* lineage 4 (GenBank accession MN200258) had a single mixed base (adenine or guanine), while the 2 other isolates of

Table 11. Pairwise comparisons of partial sequences of the 28S rRNA gene between lineages of *Crassiphiala* included in this study. Percentage differences are given above diagonal and the number of variable nucleotide positions is given below the diagonal. The 28S results are based on a 1,132 bp long alignment Abbreviations: BR, Brazil; USA, United States of America.

	***Cr.* 1** MN200253	***Cr.* 2** MN200254	***Cr.* 3** MN200257	***Cr.* 4** MN200260	***Cr.* 5** MN200261
Crassiphiala lineage 1 MN200253 USA	–	0.9%	1.1%	2.2%	1.1%
Crassiphiala lineage 2 MN200254 USA	10	–	0.2%	2.2%	0.2%
Crassiphiala lineage 3 MN200257 USA	12	2	–	2.4%	0.4%
Crassiphiala lineage 4 MN200260 BR	25	25	27	–	2.4%
Crassiphiala lineage 5 MN200261 BR	12	2	4	27	–

Crassiphiala lineage 4 (GenBank accessions MN200259, MN200260) had only guanine in this position. No other variation within lineages was detected in sequences of 28S.

Pairwise nucleotide comparisons of *cox*1 sequences among all unique *Crassiphiala* isolates are provided in Table 12. The *cox*1 sequences showed a much greater divergence among lineages (11–19.8%) which provides strong evidence that these forms are likely different species. There were no differences between the *cox*1 sequences of *Crassiphiala* lineage 4.

Discussion

The morphology of the adult *Crassiphiala* specimens included in our study conforms closely to the diagnosis of the genus provided by Niewiadomska (2002d) (Fig. 15).

While some authors (Blasco-Costa & Locke, 2017; Hernández-Mena et al., 2017; Locke et al., 2018) have noted that the Crassiphialinae may be elevated to the family level, our analysis of 28S did not support the monophyly of the Crassiphialinae with 2 clades comprising members of this subfamily being branches of a large polytomy. This is in concordance with the phylogenetic data and branch support of corresponding clades in some of the recent works, e. g., Blasco-Costa & Locke (2017) and Hernández-Mena et al. (2017) which reported a low level of

Table 12. Pairwise comparisons of partial sequences of the *cox*1 mtDNA gene between lineages of *Crassiphiala* included in this study. Percentage differences are given above diagonal and the number of variable nucleotide positions is given below the diagonal. Results are based on a 435 bp long alignment. Abbreviations: BR, Brazil; USA, United States of America.

	***Cr.* 1** MN193951	***Cr.* 2** MN193952	***Cr.* 2** MN193953	***Cr.* 3** MN193956	***Cr.* 4** MN193958	***Cr.* 5** MN193959	***Cr.* 5** MN193960
Crassiphiala lineage 1 MN193951 USA	–	12.4%	12.2%	16.3%	15.6%	14.3%	14.3%
Crassiphiala lineage 2 MN193952 USA	54	–	0.2%	13.1%	16.1%	11.3%	11.5%
Crassiphiala lineage 2 MN193953 USA	53	1	–	12.9%	15.9%	11.0%	11.3%
Crassiphiala lineage 3 MN193956 USA	71	57	56	–	19.8%	12.9%	13.1%
Crassiphiala lineage 4 MN193958 BR	68	70	69	86	–	17.0%	17.2%
Crassiphiala lineage 5 MN193959 BR	62	49	48	56	74	–	0.7%
Crassiphiala lineage 5 MN193951 BR	62	50	49	57	75	3	–

support for the Crassiphialinae. Although the authors of those publications suggested the monophyly of the Crassiphialinae, their phylogenetic trees did not provide a sufficient evidence for such conclusions. The content and systematics of the Crassiphialinae need to be carefully re-evaluated based on a detailed morphological study and additional phylogenetic analyses of its constituent taxa which is outside of the scope of this work. Based on the data obtained in the present work, particularly the demonstrated non-monophyly of the Crassiphialinae, we do not see a sufficient ground for elevating its status to the family level. Moreover, the content of the Crassiphialinae as currently recognized (Niewiadomska, 2002d) needs to be revised; likely, only *Crassiphiala* and *Uvulifer* should remain in the subfamily. However, a more detailed analysis involving a greater diversity of crassiphialine taxa and a thorough morphological study is needed to adequately address this question.

Despite the relatively low number of *Crassiphiala* lineages, our analyses (Figs 13, 14) allowed for an interesting observation that the phylogenetic relationships within the genus do not follow the geographic origin of the samples. One of the lineages from Brazil (*Crassiphiala* lineage 4) appeared on the trees as the sister group to all other members of the genus while the

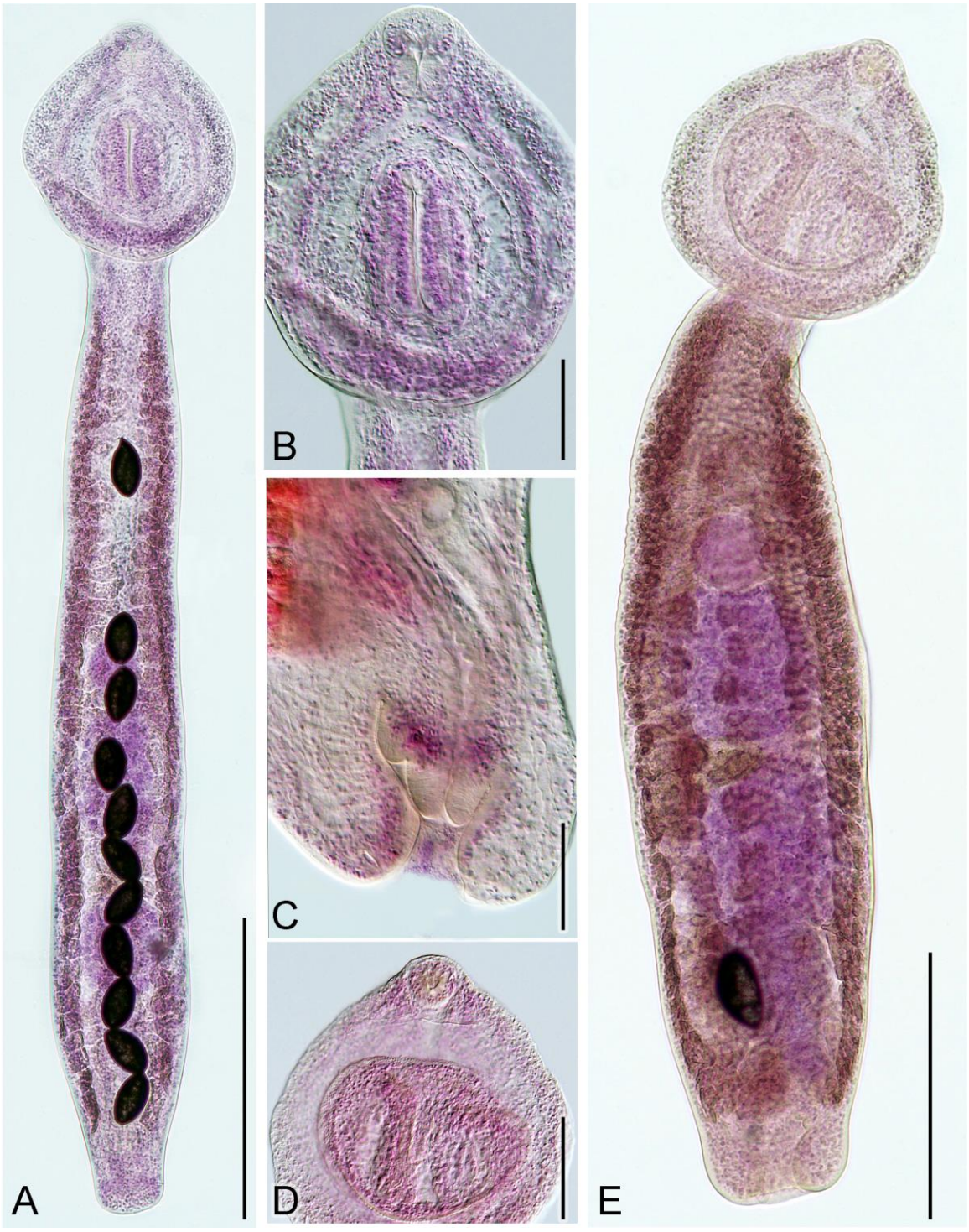

Figure 15. Adults of *Crassiphiala* lineages 4 and 5 collected from the intestines of *Megaceryle torquata* from Pantanal, Brazil. **(A)** Ventral view of whole mount of *Crassiphiala* lineage 4. **(B)** Ventral view of the prosoma of *Crassiphiala* lineage 4. **(C)** Lateral view of posterior body end of *Crassiphiala* lineage 4. **(D)** Ventral view of the prosoma of *Crassiphiala* lineage 5. **(E)** Ventral view of whole mount of *Crassiphiala* lineage 5. Scale bars: A, 500 µm; B, C, D, 100 µm; E, 300 µm. (after Achatz et al., 2019c)

branch that included the other Brazilian lineage (*Crassiphiala* lineage 5) was nested among North American isolates. The reasons for this pattern are not clear at this time. One explanation may be the relatively old evolutionary origin of *Crassiphiala* which allowed for transcontinental spread (in both directions). Another explanation could be based on the partial overlap of the geographic distribution of the typically North American *M. alcyon* with several species of kingfishers broadly distributed in the Central and South America.

This study is the first to generate DNA sequence data of adult specimens of *Crassiphiala* and the first to report *Crassiphiala* in the Neotropics. Our results demonstrated the presence of at least 5 lineages of *Crassiphiala* in the Nearctic and Neotropics. This indicates that the diversity of *Crassiphiala* was seriously underestimated and allows us to hypothesize that additional species belonging to this genus are likely to be discovered in future studies. Central and South America hold a greater potential in this respect due to the more diverse fauna of kingfishers.

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Associated publication

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CHAPTER VII

PHYLOGENETIC POSITION OF *CODONOCEPHALUS* DIESING, 1850 (DIGENEA, DIPLOSTOMOIDEA), AN UNUSUAL DIPLOSTOMID WITH PROGENETIC METACERCARIAE

Introduction

Codonocephalus Diesing, 1850 is a monotypic genus of diplostomid digeneans (Diplostomidae Poirier, 1886) and is the sole genus recognized in the subfamily Codonocephalinae Sudarikov, 1959 (Niewiadomska, 2002d). Its type-species *Codonocephalus urniger* (Rudolphi, 1819) Lühe, 1909 has a life cycle involving a lymnaeid snail as the first intermediate host and frogs (usually *Pelophylax* spp.) as the second intermediate host (Niewiadomska, 1964). In some cases, snakes (usually *Natrix* spp., rarely *Elaphe* spp.) serve as a paratenic host (Sudarikov, 1959; Sharpilo, 1976). Adult *C. urniger* parasitize ardeid wading birds in the Palearctic. Unlike most other diplostomoideans, metacercariae of *C. urniger* are progenetic and have fully-developed reproductive organs while in the intermediate/paratenic hosts (Niewiadomska, 1964, 2002d). Adult *C. urniger* differ morphologically from most other diplostomids in having an infundibular or cup-shaped prosoma (the anterior body 'region' or 'segment') with a crenulated border and lacking a clear separation between the prosoma and opisthosoma (the posterior body 'region' or 'segment'). In addition, *C. urniger* has an indistinct holdfast organ and a very strongly-developed proteolytic gland (Niewiadomska, 2002d).

The unusual adult and metacercarial morphologies have resulted in a convoluted systematic history of *Codonocephalus* (Kostadinova, 1993; Niewiadomska, 2002d). Sudarikov (1959) created the subfamily Codonocephalinae within the Strigeidae Railliet, 1919; while Dubois (1970b) placed *Codonocephalus* into the Diplostomidae within the tribe Codonocephalini Sudarikov, 1959. Yamaguti (1971) subsequently placed *Codonocephalus* within the strigeid tribe Cotylurini Dubois, 1936, while Sudarikov (1984) re-evaluated the position of *Codonocephalus* and returned it to the Diplostomidae. The genus was positioned in the subfamily Codonocephalinae within the Diplostomidae in the most recent revision of the Diplostomoidea Poirier, 1886 by Niewiadomska (2002d). While there has been a number of studies related to the biology of *Codonocephalus* (e.g., Niewiadomska, 1964; Kostadinova, 1993) and a recent surge of molecular phylogenetic studies of diplostomoideans (e.g., Blasco-Costa & Locke, 2017; Hernández-Mena et al., 2017; Achatz et al., 2019d), DNA sequence data for *C. urniger* is lacking. Herein, we present the first molecular phylogenetic analysis based on the nuclear ribosomal 28S DNA sequences to include *Codonocephalus*. We also provide mitochondrial cytochrome c oxidase subunit 1 (*cox*1) sequences for future comparative studies.

Materials & Methods

Specimens

Progenetic metacercariae of *C. urniger* were obtained from tissues and body cavity of the Marsh Frog, *Pelophylax ridibundus* (Pallas), collected in the Southern Bug River in the vicinities of the village of Pisky in Mykolaivska Oblast, Ukraine (47°8'52.24"N; 31°50'58.34"E) on 29 August 2018. Numerous live metacercariae found in *P. ridibundus* were removed from the cysts, briefly rinsed in 0.9% saline, killed with hot 70% ethanol and preserved in 70% ethanol.

Morphological vouchers have been deposited in the collection of the H. W. Manter Laboratory, University of Nebraska, Lincoln under accession number HWML-216011.

Phylogenetic analysis

Newly generated sequences were deposited in the GenBank (28S GenBank accession numbers: MN250790, MN250791; *cox*1 GenBank accession numbers: MN258113, MN258114). The phylogenetic relationships of *C. urniger* were analyzed using 28S sequences. The alignment included a newly generated sequence of *C. urniger* and previously published sequences of 14 members of the Diplostomidae, 1 member of the Proterodiplostomidae Dubois, 1936 and 12 members of the Strigeidae. *Suchocyathocotyle crocodili* (Yamaguti, 1954) (GenBank accession MK650450) was selected as outgroup in the 28S analysis based on the topology presented by Achatz et al. (2019d).

Phylogenetic analysis was conducted using Bayesian inference (BI) as implemented in MrBayes Ver. 3.2.6 software (Ronquist & Huelsenbeck, 2003). The general time-reversible model with estimates of invariant sites and gamma-distributed among-site variation (GTR + I + G) was identified as the best-fitting nucleotide substitution model using MEGA7 software (Kumar et al., 2016). The BI analysis was performed using MrBayes software as follows: Markov chain Monte Carlo (MCMC) chains were run for 3,000,000 generations with sample frequency set at 1,000. Log-likelihood scores were plotted and only the final 75% of trees were used to produce the consensus trees by setting the "burn-in" parameter at 750. This number of generations was considered sufficient because the SD dropped below 0.01. The trees were visualized in FigTree ver. 1.4 software (Rambaut, 2016) and annotated in Adobe Illustrator®.

Results

We found *C. urniger* in 34 out of 96 (35.4%) *P. ridibundus* examined in the vicinity of the village Pisky in Ukraine, with the intensity of infection ranging from 1 to 262 metacercariae in a single frog. The morphology of our progenetic metacercariae closely conformed to the description of *C. urniger* (Fig. 16). The holdfast organ of our *C. urniger* specimens appeared as a weakly-developed structure lacking the sucker-like appearance typical of other diplostomids while the proteolytic gland located at the base of the holdfast organ was very well-developed. The metacercariae were usually concentrated under the skin and around organs in the anterior part of the body (Fig. 16F).

No differences were detected among 28S sequences of the 2 extracted isolates of *C. urniger*, while only 1 base out of 598 (0.17%) was different between the *cox*1 sequences of the 2 isolates.

Upon trimming to the length of the shortest sequence the 28S alignment was 1,161 bp long. The phylogenetic tree resulting from the BI analysis (Fig. 17) demonstrated the non-monophyletic nature of the Diplostomidae and Strigeidae, similar to the results of several previous studies (e.g., Blasco-Costa & Locke, 2017; Hernández-Mena et al., 2017; Achatz et al., 2019d). The Proterodiplostomidae formed a very poorly supported sister clade to the large polytomy including the Diplostomidae and the Strigeidae (Fig. 17). Members of the Diplostomidae formed 7 strongly supported clades: 1) *Bolbophorus* Dubois, 1935 + *Ornithodiplostomum* Dubois, 1936 + *Posthodiplostomum* Dubois, 1936 (85%), 2) *Uvulifer* (100%), 3) *Hysteromorpha* Lutz, 1931, 4) *Austrodiplostomum* Szidat & Nani, 1951 + *Diplostomum* von Nordmann, 1832 + *Tylodelphys* Diesing, 1850 (100%), 5) *Alaria* Schrank, 1788 (99%), 6) *Neodiplostomum* Railliet, 1919 and 7) *Codonocephalus* (Fig. 17). Members of

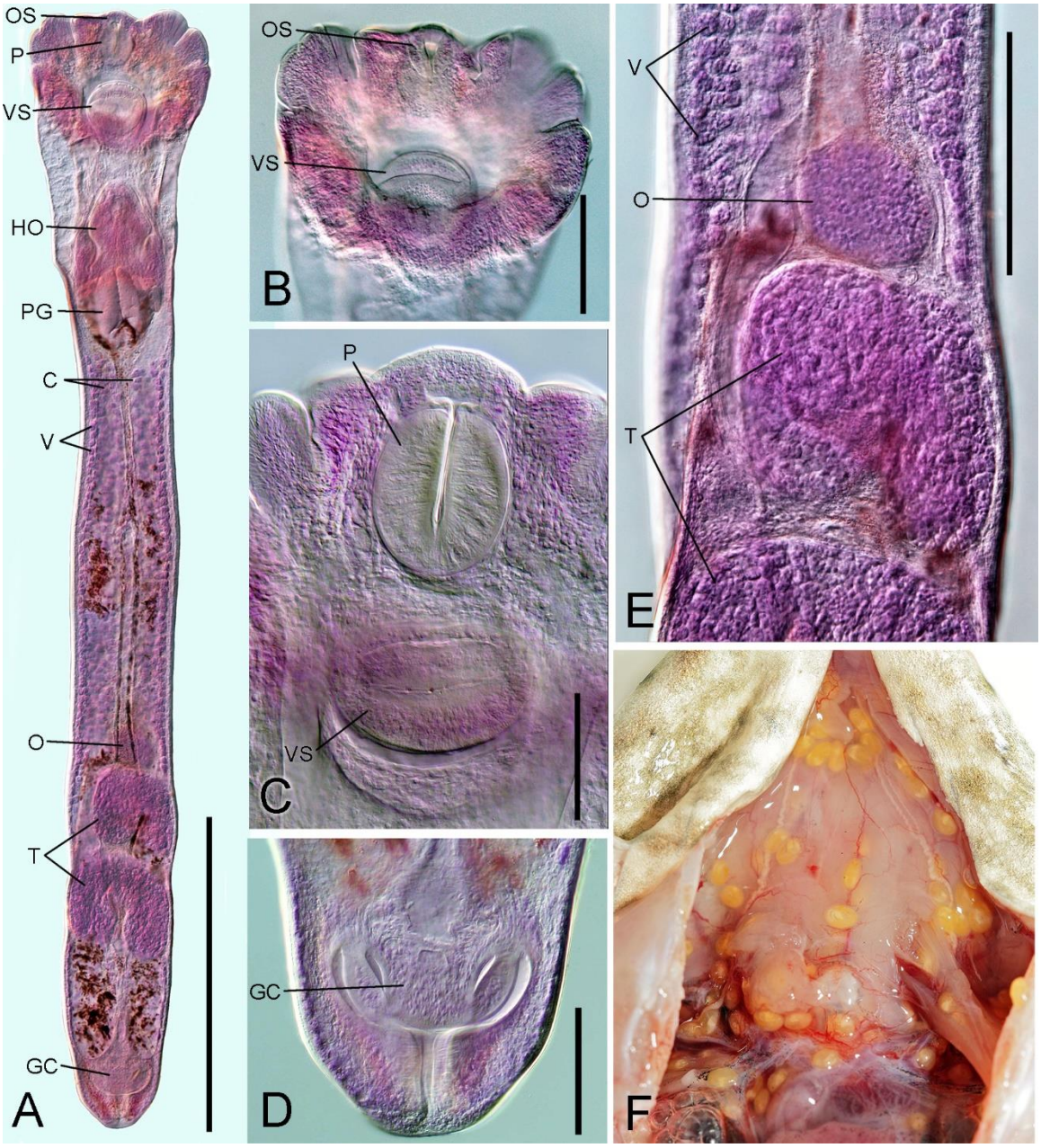

Figure 16. Progenetic metacercaria of *Codonocephalus urniger*. Note the fully developed reproductive system organs. (**A**) Ventral view of whole mount; (**B**) Ventral view of the prosoma with crenulated border; (**C**) Ventral view of prosoma; (**D**) Ventral view of posterior body end; (**E**) Ventral view of opisthosoma with gonads; (**F**) Dissected frog infected with *C. urniger*. Scale bars: A, 1,000 µm; B, 200 µm; C, D, 100 µm; E, 250 µm. Abbreviations: C, ceca; GC, genital cone; HO, holdfast organ; O, ovary; OS, oral sucker; P, pharynx; PG, proteolytic gland; T, testis; V, vitellarium; VS, ventral sucker. (after Achatz et al., 2019b)

the Strigeidae formed 3 strongly supported clades. The first clade (100%) included *Apharyngostrigea* Ciurea, 1927 + *Strigea* Abildgaard, 1790 + *Apatemon* Szidat, 1928+ *Australapatemon* Sudarikov, 1959; the second clade (98%) included *Cotylurus* Szidat, 1928 + *Ichthyocotylurus* Odening, 1969; the third clade only included *Cardiocephaloides* Sudarikov, 1959 (100%).

Discussion

As mentioned in the introduction, this genus has been moved between the Diplostomidae and the Strigeidae multiple times depending on the views of a particular author and the morphological characters used. In addition, Kostadinova (1993) demonstrated that the cercarial chaetotaxy of *C. urniger* is more similar to that found in diplostomids (notably *Diplostomum* spp.) than in strigeids. Our phylogenetic tree based on 28S sequences demonstrated that *C. urniger* forms yet another unique branch in the polytomy encompassing the Diplostomidae, Strigeidae, and Proterodiplostomidae, thus adding to the already existing complexity and posing a question regarding the taxonomic rank of this highly unusual digenean.

The presence of a progenetic metacercaria in *Codonocephalus* along with its morphology atypical for diplostomids (Figs 16, 18) are reflected in its phylogenetic position as an independent branch among other major lineages of the highly diverse Diplostomoidea (Fig. 17). The extreme level of non-monophyly of the currently accepted Diplostomidae and other Diplostomoideans requires a detailed re-evaluation of the group as a whole, which is beyond the scope of the present study. The *cox*1 sequences generated in this study can be used for comparative purposes in the future when more sequence data from a greater geographic and host range will become available. Based on the broad geographic distribution of *C. urniger*, it cannot

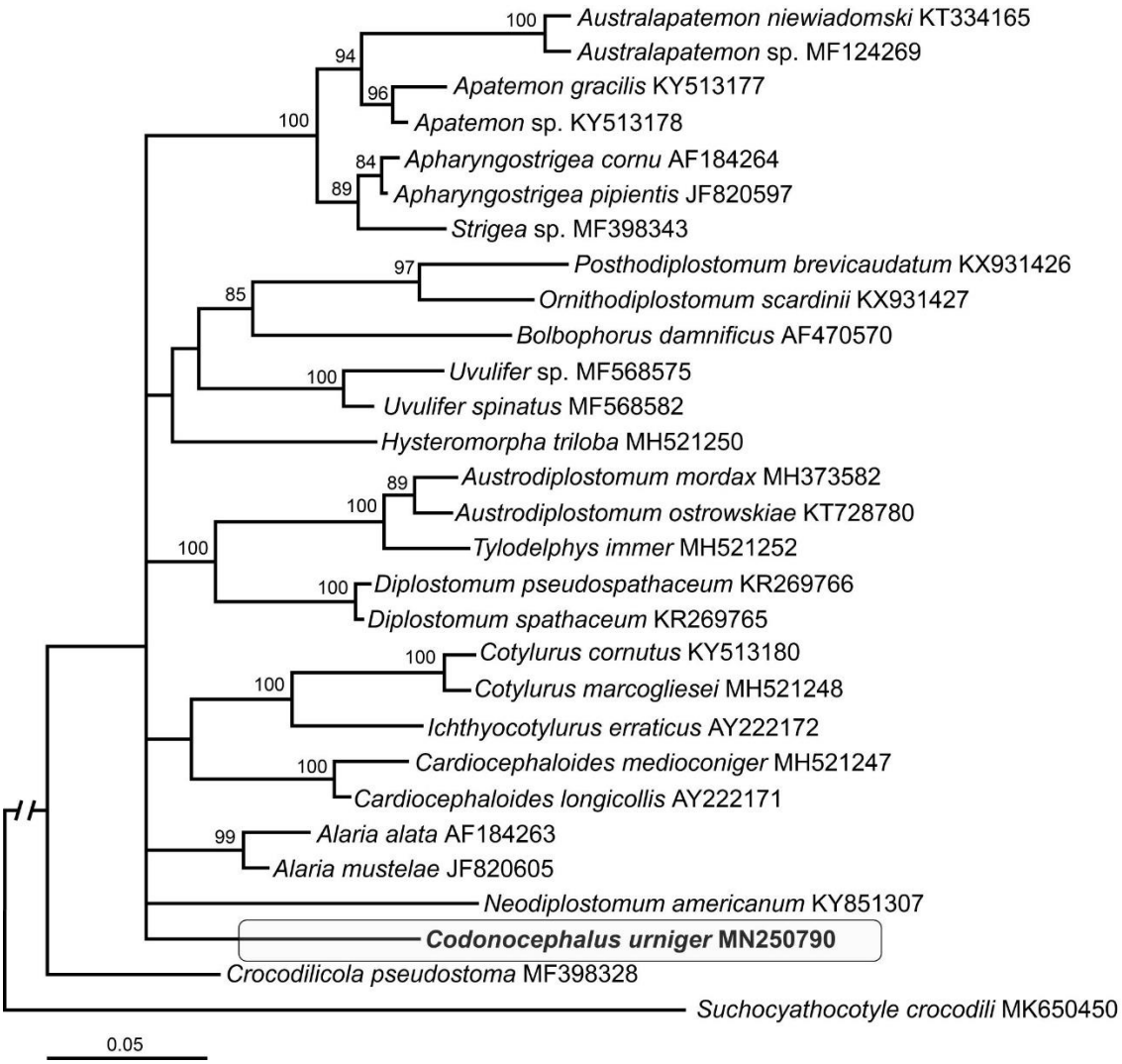

Figure 17. Phylogenetic position of *Codonocephalus urniger* within the Diplostomoidea based on Bayesian Inference (BI) analysis of partial 28S rRNA gene sequences. Bayesian Inference posterior probability values lower than 80% (BI) are not shown. The new sequence obtained in this study is in bold. The gray box surrounds *Codonocephalus*. Scale bar indicates number of substitutions per site. GenBank accession numbers are provided after the names of all species. (after Achatz et al., 2019b)

be ruled out that this species may represent a complex of species or at least show a pronounced genetic variability as was the case with other diplostomoidean digeneans (e.g., Sereno-Uribe et al., 2019)

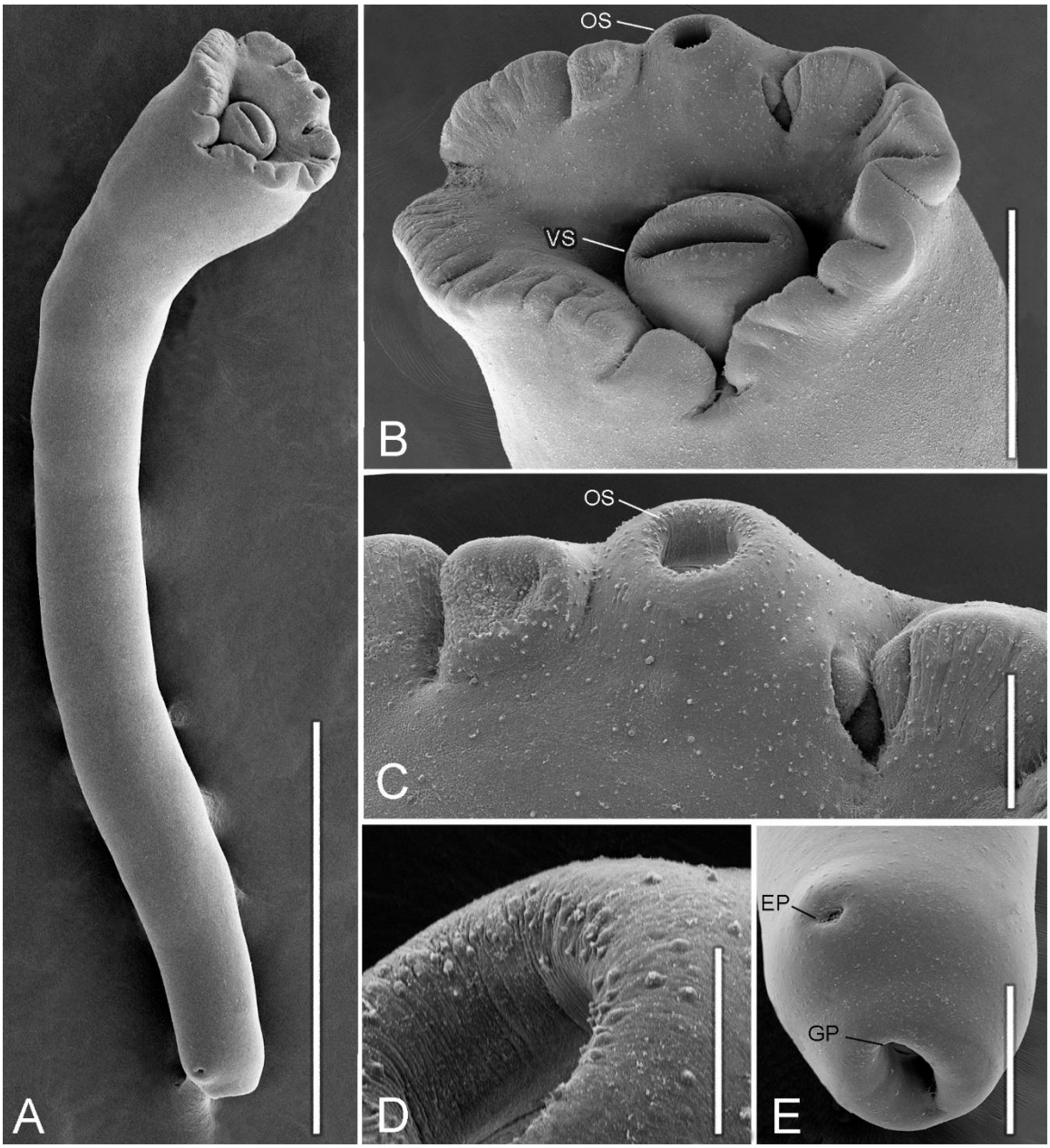

Figure 18. Scanning electron micrographs of *Codonocephalus urniger*. (**A**) Entire specimen, ventral view; (**B**) Ventral view of the prosoma with crenulated margins. (**C**) Ventral view of prosoma; (**D**) Oral sucker with numerous papillae; (**E)** Ventral view of posterior body end. Scale bars: A, 1,000 µm; B, 200 µm; C, 50 µm; D, 25 µm; E, 100 µm. Abbreviations: OS, oral sucker; EP, excretory pore; GP, genital pore; VS, ventral sucker. (after Achatz et al., 2019b)

Acknowledgments

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Associated publication

Achatz, T. J., Dmytrieva, I., Kuzmin, Y. and Tkach, V. V. 2019b. Phylogenetic position of *Codonocephalus* Diesing, 1850 (Digenea, Diplostomoidea), an unusual diplostomid with progenetic metacercariae. Journal of Parasitology 105: 821–826.

CHAPTER VIII

PHYLOGENETIC POSITION OF *SPHINCTERODIPLOSTOMUM* DUBOIS, 1936 (DIGENEA: DIPLOSTOMOIDEA) WITH DESCRIPTION OF A SECOND SPECIES FROM PANTANAL, BRAZIL

Introduction

Sphincterodiplostomum Dubois, 1936 is a monotypic genus of diplostomoidean digeneans (Diplostomidae Poirier, 1886; Diplostominae Poirier, 1886), which parasitize the intestines of their avian definitive hosts in the Neotropics (Niewiadomska, 2002d; Lunaschi & Drago, 2006). The type species *Sphincterodiplostomum musculosum* Dubois, 1936 was originally described by Dubois (1936b, 1938) based on immature specimens from agami heron *Agamia agami* (Gmelin) collected in Brazil. Lunaschi & Drago (2006) have described fully mature adult specimens of the species from great egret *Ardea alba* Linnaeus in Argentina. *Sphincterodiplostomum musculosum* is most easily differentiated from other members of the Diplostomidae based on the presence of a well-developed, dorsal, tubular invagination in the opisthosoma with a muscular sphincter (Niewiadomska, 2002d; Lunaschi & Drago, 2006).

The complete life cycle of *S. musculosum* has not been demonstrated, however, *S. musculosum* is known to utilize a wide diversity of fish as second intermediate hosts, and has been previously collected from avian definitive hosts (e.g., Dubois, 1936b; Lunaschi & Drago, 2006; Rocha et al., 2015; Delgado et al., 2017). Adult *S. musculosum* have been

rarely collected (e.g., Lunaschi & Drago, 2006), whereas, metacercariae have been reported in several studies of Neotropical fish helminths (e.g., Szidat, 1969, Zago et al., 2013; Rocha et al., 2015; Delgado et al., 2017). To date, no DNA sequence data have been published for *S. musculosum*. Herein, we provide partial 28S rRNA and cytochrome *c* oxidase (*cox*1) mtDNA gene sequences of *S. musculosum* and a new *Sphincterodiplostomum* species collected from avian and crocodilian hosts. The 28S DNA sequence data were used to infer the phylogenetic position of *Sphincterodiplostomum* spp. among other major diplostomoidean lineages. The sequences of *cox*1 were used for reliable *Sphincterodiplostomum* species differentiation.

Materials & Methods

Specimens

Vertebrate hosts were collected in Pantanal, Fazenda Retiro Novo, Municipality of Poconé, Mato Grosso State, Brazil. Mature and immature adult specimens of *S. musculosum* were obtained from cocoi heron *Ardea cocoi* Linnaeus, black-collared hawk *Busarellus nigricollis* (Latham), green kingfisher *Chloroceryle americana* (Gmelin) and yacare caiman *Caiman yacare* (Daudin). In addition, mature adult specimens of the new *Sphincterodiplostomum* species were collected from *B. nigricollis* (Table 13). Specimens for morphological study were stained with an aqueous alum carmine and permanently mounted according to Lutz et al. (2017). Type and voucher specimens are deposited in the collection of the Harold W. Manter Laboratory (HWML), University of Nebraska State Museum, Lincoln, Nebraska, U.S.A.

Phylogenetic analysis

Newly obtained and previously published sequences were initially aligned using ClustalW implemented in MEGA7 software (Kumar *et al.*, 2016). The position of *Sphincterodiplostomum* spp. among major diplostomoidean lineages was studied using an alignment of 28S, which included newly generated sequences of both *Sphincterodiplostomum* species and previously published sequences of 16 members of the Diplostomidae, 2 members of the Proterodiplostomidae Dubois, 1936 and 12 members of the Strigeidae Railliet, 1919. *Suchocyathocotyle crocodili* (Yamaguti, 1954) was selected as the outgroup based on the topology presented by Achatz et al. (2019d).

The phylogenetic analysis was conducted using Bayesian inference (BI) as implemented in MrBayes Ver. 3.2.6 software (Ronquist & Huelsenbeck, 2003). The best-fitting nucleotide substitution model identified by MEGA7 was the general time-reversible model with estimates of invariant sites and gamma-distributed among-site variation (GTR+ I + G). The BI analysis was performed using MrBayes software as follows: Markov chain Monte Carlo (MCMC) chains were run for 3,000,000 generations with sample frequency set at 1,000. Log-likelihood scores were plotted and only the final 75% of treeswere used to produce the consensus trees. The number of generations for each analysis was considered sufficient as the standard deviation stabilized below 0.01. The pairwise comparisons of *Sphincterodiplostomum* isolates were performed with assistance of MEGA7 software.

Table 13. List of *Sphincterodiplostomum* isolates sequenced in this study including their hosts and GenBank accession numbers. HWML: Harold W. Manter Laboratory, Lincoln, Nebraska, U.S.A. All specimens were collected at Fazenda Retiro Novo, Municipality of Poconé, Mato Grosso State, Brazil.

Digenean taxa	Host species	Museum No.	Accession numbers	
			28S	***cox*1**
Sphincterodiplostomum joaopinhoi n. sp.	*Busarellus nigricollis*	HWML-216379, 216380	MW411441, MW411442	MW410851, MW410852
Sphincterodiplostomum musculosum	*Ardea cocoi*	HWML-216381	MW411443	MW410853
S. musculosum	*Chloroceryle americana*	HWML-216382	–	MW410854
S. musculosum	*B. nigricollis*	HWML-216383	MW411444	MW410855
S. musculosum	*Caiman yacare*	HWML-216384	MW411445	MW410856

Results

Description of new species

Sphincterodiplostomum joaopinhoi n. sp.

(Figs 19–21)

Description [Based on 2 adult specimens; see Figs 19, 20a–e, 21a–e]. Body 978–1,259 long, consisting of distinct prosoma and opisthosoma; prosoma elliptical, 580–766 long, with maximum width at level of holdfast organ, 428–460; opisthosoma cylindrical, 398–493 long, 226–241 wide. Prosoma: opisthosoma length ratio 1.5–1.6; Prosoma: opisthosoma width ratio 1.8–2. Forebody 347–451 long, 35–36% of body length. Minuscule tegumental spines covering most of prosoma, absent between anterior margin of oral sucker and posterior margin of pseudosuckers; spines scale-like with several small digitiform projections at posterior edge (Fig. 21d). Opisthosoma with a tubular invagination with muscular sphincter at level of posterior testis. Oral sucker terminal, oval, 64–72 × 49–56. Pseudosuckers 75–82 × 66–78. Ventral sucker with minute spines covering its base, 93–98 × 98–108, located near 60% of prosoma length; oral: ventral sucker width ratio 0.5. Holdfast organ immediately posterior to ventral sucker; subspherical or oval with ventral muscular portion, 118–128 × 110–168. Proteolytic gland at

base of holdfast organ, bilobed, 43 × 79. Prepharynx 24 long. Pharynx oval, 76 × 46. Esophagus 54–80 long. Cecal bifurcation in anterior third of prosoma. Ceca slender, extending to near posterior end of opisthosoma.

Testes 2, in tandem, lobate; anterior testis asymmetrical, 68–112 × 156–203; posterior testis symmetrical, horseshoe-shaped with anterior isthmus, 152–171 × 165–209. Seminal vesicle folded, posterior to isthmus of posterior testis; terminal efferent duct of seminal vesicle joins dorsal side of metraterm to form short hermaphroditic duct.

Ovary pretesticular, near prosoma-opisthosoma junction, subspherical or slightly transversely oval 55–60 × 64–75. Oötype and Mehlis' gland inter-testicular. Laurer's canal not observed. Vitelline follicles distributed as 2 lateral bands extending posteriorly from approximately the level of the ventral sucker to near the posterior end of the body, lateral bands sporadically confluent. Vitelline follicles absent in the first 47–68% of prosoma and last 15–20% of opisthosoma. Vitelline reservoir inter-testicular. Uterus ventral to gonads, extending anteriorly to near junction of prosoma and opisthosoma before turning and extending posteriorly. Uterus contains up to 8 eggs (74–83 × 42–53).

Genital pore subterminal, on dorsal side, muscular. Excretory vesicle not well-observed. Excretory pore subterminal, on dorsal side.

Taxonomic summary

Type-host. Busarellus nigricollis (Latham) (Accipitriformes: Accipitridae).

Type-locality. Pantanal, Fazenda Retiro Novo, Municipality of Poconé, Mato Grosso State, Brazil (16º21'53''S, 56º17'31''W).

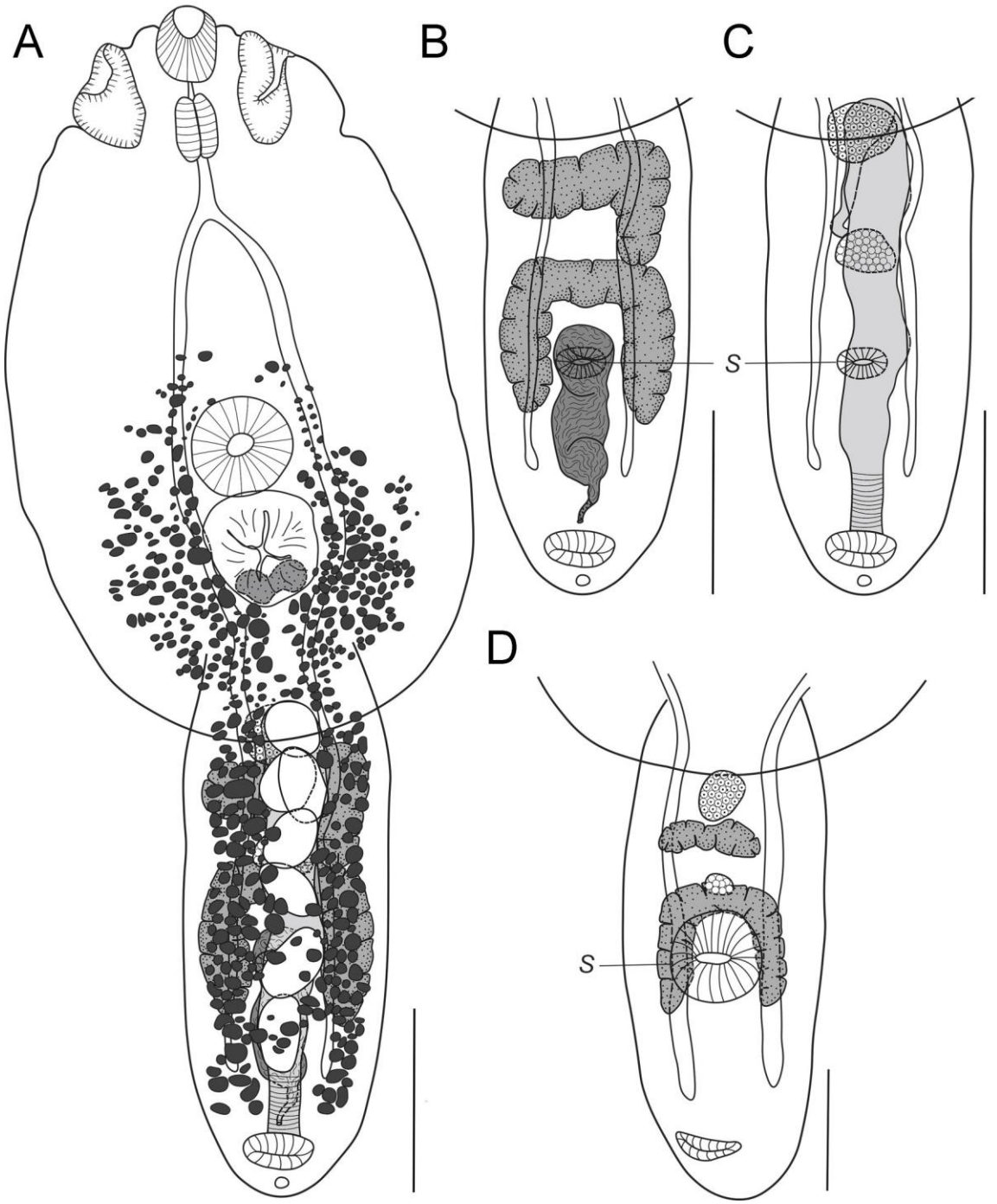

Figure 19. Line drawings of *Sphincterodiplostomum joaopinhoi* n. sp. **(A)** Holotype, ventral view; **(B)** Male reproductive system of holotype, ventral view of opisthosoma; **(C)** Female reproductive system of holotype, ventral view of opisthosoma, vitellarium and eggs omitted; **(D)** Paratype, immature specimen, dorsal view. Scale bars: A, B, C, 200 µm; D, 100 µm. Abbreviation: S, dorsal muscular sphincter associated with the tubular invagination of the opisthosoma. (after Achatz et al., 2021)

Type-material. The type series consists of 2 mature and 4 immature adult specimens deposited in the HWML. Holotype: HWML-216379, labeled ex. *Busarellus nigricollis*, small intestine, Pantanal, Fazenda Retiro Novo, Municipality of Poconé, Mato Grosso State, Brazil, 9 June 2017, coll. A. Fecchio. Paratypes: HWML-216380 (lot of 4), labels identical to the holotype.

Site in host: Small intestine.

ZooBank registration. The Life Science Identifier (LSID) for *Sphincterodiplostomum joaopinhoi* n. sp. is urn:lsid:zoobank.org:act:DB466D7B-EED6-4959-9EB9-E3C34A3D4885.

Etymology. The species is named after Dr. Joao B. Pinho (Laboratório de Ecologia de Aves, Federal University of Mato Grosso, Cuiabá, Brazil) in recognition of his contributions into the knowledge of avifauna of Pantanal and his invaluable assistance with collecting specimens reported in this work.

Remarks

The new species clearly belongs to *Sphincterodiplostomum* based on the presence of a well-developed dorsal tubular invagination in the opisthosoma with a muscular sphincter, along with the results of our molecular phylogenetic analysis (Fig. 22). The differential diagnosis below compares the new species with the description of adult *S. musculosum* by Lunaschi & Drago (2006) as they were the first to describe mature adult specimens. It is worth noting that specimens described by Lunaschi & Drago (2006) were contracted as stated by the authors and evident based on their illustrations. As we had only a single fully mature ovigerous specimen, we do not provide a description of *S. musculosum*. For the same reason we do not provide a differential diagnosis based on our material, except for the tegumental spine structure and body size which is skewed in the description by Lunaschi & Drago (2006) due to contraction.

Figure 20. Specimens of *Sphincterodiplostomum* species from Pantanal, Brazil. **(A)** Holotype of mature adult *S. joaopinhoi* n. sp., ventral view; **(B, C)** Paratypes of immature *S. joaopinhoi* n. sp. at different stages of development, ventral views; **(D, E)** Opisthosoma of *S. joaopinhoi* n. sp., dorsal views; **(F)** Mature adult *S. musculosum* from *Ch. americana*, ventral view, hologenophore; **(G)** Immature *S. musculosum* from *Ar. cocoi*, dorsal view; **(H)** Immature *S. musculosum* from *Ca. yacare*, dorsal view. Scale bars: A, B, C, 250 µm; D, E, 150 µm; F, G, H, 500 µm. Abbreviation: *S*, sphincter surrounding tubular invagination characteristic of *Sphincterodiplostomum* species. (after Achatz et al., 2021)

Sphincterodiplostomum joaopinhoi n. sp. can be easily distinguished from *S. musculosum* based on the anterior extent of vitelline follicles (limited to near level of ventral sucker in the new species vs reaching near the level of the cecal bifurcation in *S. musculosum*) (Figs 19, 20).

Sphincterodiplostomum joaopinhoi n. sp. is smaller than heat-killed, properly fixed adult specimen of *S. musculosum* in our material (body length 978–1,259 in the new species vs body length 1,821 in *S. musculosum*). Even immature heat-killed specimens of *S. musculosum* in our material are substantially larger (body length 2,145–2,593) than the new species (body length 978–1,259). *Sphincterodiplostomum joaopinhoi* n. sp. differs from *S. musculosum* described by Lunaschi & Drago (2006) by a much smaller prosoma width (428–460 in *S. joaopinhoi* n. sp. vs 754–1,115 in *S. musculosum*), smaller oral sucker (64–72 × 49–56 in the new species vs 92–108 × 63–106 in *S. musculosum*), shorter pseudosuckers (75–82 in *S. joaopinhoi* n. sp. vs 101–150 in *S. musculosum*), smaller holdfast organ (118–128 × 110–168 in the new species vs 143–314 × 217–580 in *S. musculosum*), shorter pharynx (76 in *S. joaopinhoi* n. sp. vs 111–140 in *S. musculosum*) and wider anterior testis (156–203 in the new species vs 95–113 in *S. musculosum*). Our SEM study demonstrated that the structure of tegumental spines of *S. joaopinhoi* n. sp. (Fig. 21d) also differs from that in *S. musculosum* (Fig. 21f, g). The spines of *S. joaopinhoi* n. sp. are scale-like and have several digitiform projections at the posterior edge of each spine (Fig. 21d), whereas spines of *S. musculosum* are not scale- like and lack such projections (Fig. 21f, g). *Sphincterodiplostomum joaopinhoi* n. sp. differs from *S. musculosum* by 0.7% (8 bases out of 1,193) in the partial sequences of 28S gene and by 10.6–11.7% (58–64 bases out of 545) in the partial sequences of *cox*1 gene.

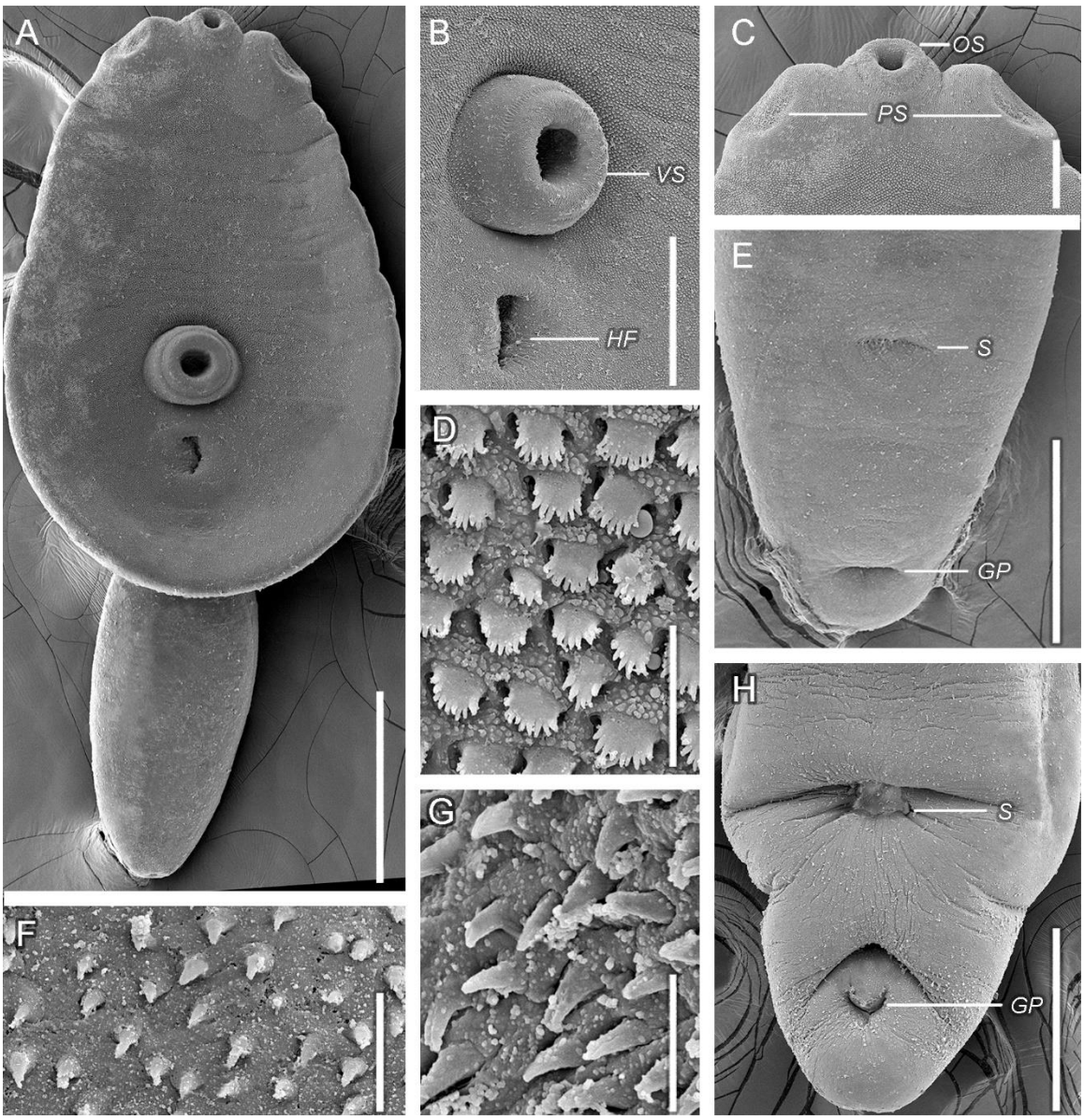

Figure 21. Scanning electron micrographs of *Sphincterodiplostomum* spp. **(A)** Entire specimen of *S. joaopinhoi* n. sp., ventral view; **(B)** Ventral sucker and holdfast organ of *S. joaopinhoi* n. sp., ventral view; inset shows minute spines at the base of the ventral sucker; **(C)** Anterior end of prosoma of *S. joaopinhoi* n. sp., ventral view; **(D)** Tegumental spines with digitiform projections of *S. joaopinhoi* n. sp.; **(E)** Posterior end of opisthosoma of *S. joaopinhoi* n. sp., dorsal view, note the sphincter surrounding tubular invagination characteristic of *Sphincterodiplostomum* species; **(F, G)** Tegumental spines of *S. musculosum*; **(H)** Posterior end of opisthosoma of *S. musculosum*, dorsal view. Scale bar: A, 200 µm; B, 75 µm; C, 70 µm; D, 5 µm; E, 100 µm; F, 10 µm; G, 5 µm; H, 250 µm. Abbreviations: GP, genital pore; HF, holdfast organ; OS, oral sucker; PS, pseudosucker; S, sphincter surrounding tubular invagination; VS, ventral sucker. (after Achatz et al., 2021)

Molecular phylogeny

After trimming to the length of the shortest sequence, the alignment of 28S was 1,117 bases long; 2 sites were excluded due to indels. The topology of the Diplostomidae and Strigeidae in the phylogeny resulting from our analysis of 28S (Fig. 22) was similar to other recent molecular phylogenetic analyses of the group (e.g., Blasco-Costa & Locke, 2017; Hernández-Mena et al., 2017; Locke et al., 2018; Achatz et al., 2019b–d, 2020b; Pérez-Ponce de León & Hernández-Mena, 2019; Queiroz et al., 2020; Tkach et al., 2020). Importantly, both the Diplostomidae and Strigeidae were non-monophyletic. Both included representatives of the Proterodiplostomidae formed a strongly supported (97%) clade. Both members of *Sphincterodiplostomum* formed a strongly supported (100%) clade within a polytomy. This polytomy included 3 other clades of diplostomids and 1 well-supported clade of strigeids (Fig. 22). The 3 other clades of diplostomids included (1) an unsupported clade of *Alaria* Schrank, 1788 + a 99% supported clade of [*Diplostomum* von Nordmann, 1832 + *Tylodelphys* Diesing, 1850 + *Austrodiplostomum* Szidat et Nani, 1951]; (2) *Codonocephalus* Diesing, 1850; (3) *Neodiplostomum* Railliet, 1919. *Hysteromorpha triloba* (Rudolphi, 1819) was part of a separate, unsupported and unresolved polytomy (Fig. 22).

Genetic variation

Pairwise nucleotide comparisons of partial 28S sequences revealed 0.7% difference (8 bases out of 1,193) between the *Sphincterodiplostomum* species/species-level lineages. No intraspecific variation was detected among the partial 28S sequences of either species.

The pairwise comparisons of partial *cox*1 sequences demonstrated 10.6–11.7% difference (58–64 bases out of 545) between the two *Sphincterodiplostomum* species. In contrast with the

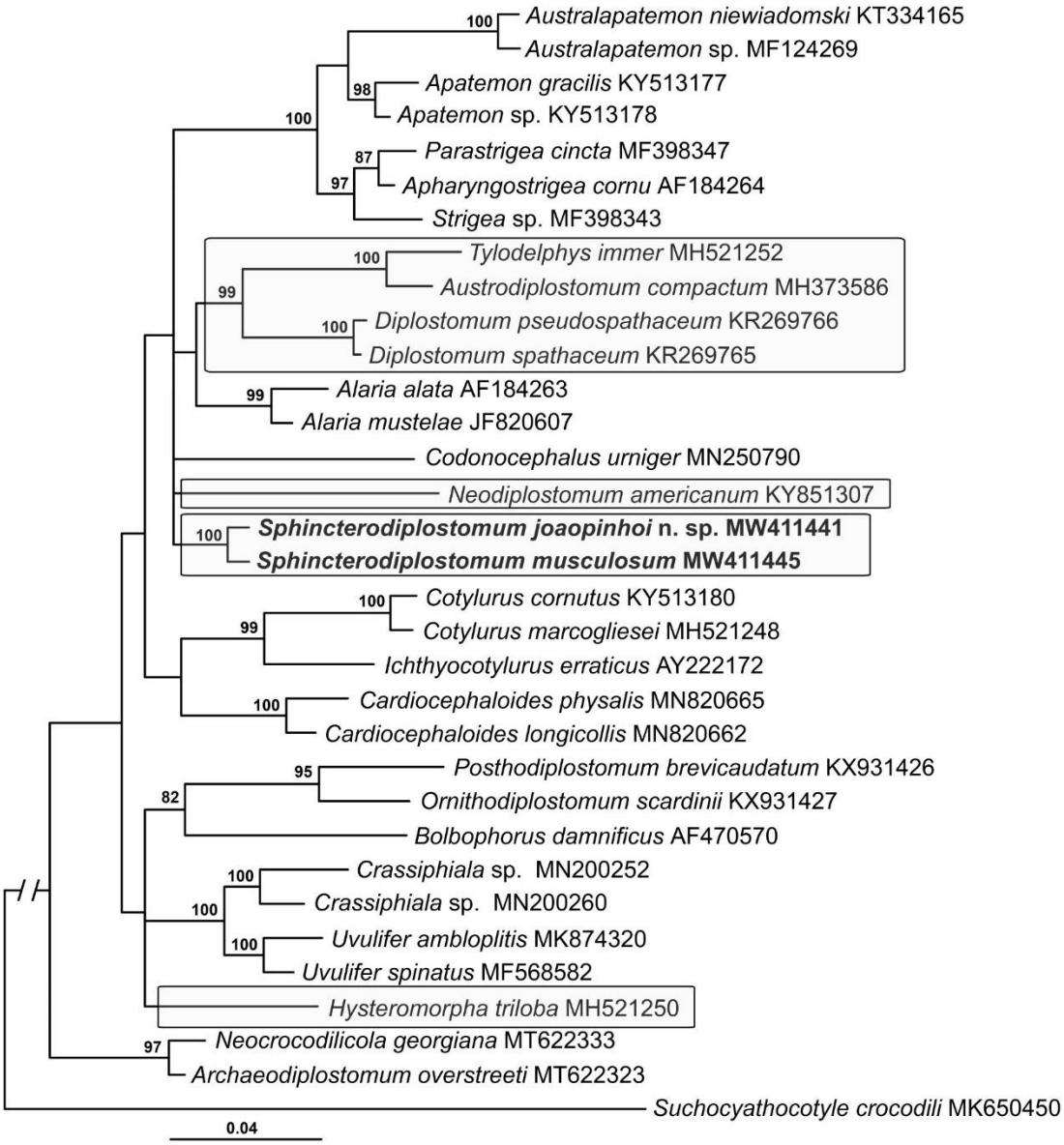

Figure 22. Phylogenetic position of *Sphincterodiplostomum* spp. within the Diplostomoidea based on Bayesian Inference (BI) analysis of partial 28S rRNA gene sequences. Members of the subfamily Diplostominae as currently recognized are indicated by the shaded rectangles. Bayesian Inference posterior probability values lower than 80% (BI) are not shown. The new sequences obtained in this study are in bold. Scale bar indicates number of substitutions per site. GenBank accession numbers are provided after the names of all species. (after Achatz et al., 2021)

28S sequences, the *cox*1 sequences demonstrated 1.3–2.6% (7–14 basesout of 545) intraspecific variation in *S. musculosum* and 0.6% (3 bases out of 545) intraspecific variation in *S. joaopinhoi* n. sp.

Discussion

This study adds a second species to the previously monotypic *Sphincterodiplostomum*. According to Niewiadomska (2002d), *Sphincterodiplostomum* belongs to the subfamily Diplostominae which has been since demonstrated to be clearly non-monophyletic (Fig. 22; e.g., Blasco-Costa & Locke, 2017; Locke et al., 2018; Achatz et al., 2019b–d; Achatz et al., 2020b; Queiroz et al., 2020; Tkach et al., 2020). Both *Sphincterodiplostomum* species possess a well-developed, dorsal tubular invagination in the opisthosoma with a muscular sphincter which is absent in other members of the Diplostominae. This fact, along with the molecular phylogenetic analysis placing the genus in its own clade with no evidence of close relationships with any other group within a polytomy, demonstrates that *Sphincterodiplostomum* represents a unique evolutionary lineage that likely evolved in South America. Whereas this evidence may be sufficient to erect a new subfamily (or family) for *Sphincterodiplostomum,* we feel that such an action would be premature until a detailed re-evaluation of all non-monophyletic members of the Diplostominae will be undertaken. With the results of this study, 28S DNA sequences are only available for 6 of the 14 genera within the Diplostominae. Thus, it is not known how inclusion of DNA sequences of the remaining Diplostominae genera may affect the resulting topology and our understanding of the relationships among all members of the subfamily.

The intrageneric pairwise nucleotide comparisons of partial 28S (0.7%) and *cox*1 (10.6–11.7%) sequences of *Sphincterodiplostomum* spp. are similar to the levels of intrageneric variation demonstrated within other diplostomoidean genera (28S: 0–4.4%; *cox*1: 3.4–19.8%; see Achatz et al., 2020b and references therein; Tkach et al., 2020).

Our mature and immature adult specimens of *S. musculosum* (Fig. 20f–h) conform closely to the original description of *S. musculosum* from *Ag. agami* by Dubois (1936b; 1938)

and redescription based on specimens from *Ar. alba* by Lunaschi & Drago (2006). Both immature and mature specimens of *S. musculosum* in our material were more similar to immature specimens described by Dubois (1936b, 1938), and were substantially longer than the contracted specimens described by Lunaschi & Drago (2006). The body length of our specimens of *S. musculosum* ranged between 1,821–2,593 despite most of them being immature, whereas Dubois (1936b, 1938) described his immature specimens to be up to 2,900 long. In contrast, the body length of the contracted specimens described by Lunaschi & Drago (2006) ranged between 919–1,329. This provides additional evidence that *S. musculosum* is a substantially larger digenean than *S. joaopinhoi* n. sp. Lunaschi & Drago (2006) described the tegument of *S. musculosum* as smooth. However, the tegument on the prosoma of our specimens is armed with spines (Fig. 21f, g). The contradiction is explained by the extremely small size of the tegumental spines, which are difficult to observe under a light microscope.

This is the first report of *S. musculosum* from *Ar. cocoi*, *B. nigricollis* (or any raptor), *Ch. americana* (or any kingfisher) and *Ca. yacare* (or any crocodilian). We assume that the infection of *Ca. yacare* was accidental based on the presence of only immature specimens and the lack of any previous reports of *S. musculosum* in crocodilians. Caimans share both habitat and diet with fish-eating birds, thus making accidental infection possible. The fact that the specimens were collected during an extremely hot time of the year from a caiman in a small, shallow water body likely explains why these digeneans, normally parasitic in birds, underwent some growth and development in a cold-blooded vertebrate.

Sphincterodiplostomum joaopinhoi n. sp. is the second member of the genus and the first *Sphincterodiplostomum* species to be reported or described from *B. nigricollis*. While we did find *S. musculosum* in studied *B. nigricollis*, we did not find any fully mature specimens. It

cannot be excluded that some previous reports of metacercariae of *S. musculosum* and unidentified *Sphincterodiplostomum* sp. from a variety of Neotropical fish may actually be *S. joaopinhoi* n. sp. The larvae of the two species are likely morphologically similar as larvae, as is the case for many other diplostomoideans; therefore, molecular identification of *Sphincterodiplostomum* metacercariae is recommended in the future. We hypothesize that the genus *Sphincterodiplostomum* contains additional not yet described species as has been recently demonstrated for several other diplostomoidean genera, such as *Crassiphiala* Van Haitsma, 1925, *Hysteromorpha* Lutz, 1931 and *Uvulifer* Yamaguti, 1934 (e.g., Locke et al., 2018; López-Jiménez et al., 2018; Achatz et al., 2019a, c).

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Científico e Tecnológico (F. T. V. Melo, grant number 431809/2018-6 Universal research fellowship number 304955/2018-3).

Associated publication

Achatz, T. J., Bell, J. A., Melo, F. T. V., Fecchio, A. and Tkach, V. V. 2021. Phylogenetic position of *Sphincterodiplostomum* Dubois, 1936 (Digenea: Diplostomoidea) with description of a second species from Pantanal, Brazil. Journal of Helminthology 95: e6.

CHAPTER IX

PHYLOGENETIC RELATIONSHIPS OF *CARDIOCEPHALOIDES* SPP. (DIGENEA, DIPLOSTOMOIDEA) AND THE GENETIC CHARACTERIZATION OF *CARDIOCEPHALOIDES PHYSALIS* (LUTZ, 1926) FROM MAGELLANIC PENGUIN, *SPHENISCUS MAGELLANICUS* (FORSTER), IN CHILE

Introduction

Cardiocephaloides Sudarikov, 1959 is a small, but essentially cosmopolitan genus of strigeid digeneans (Strigeidae Railliet, 1919) parasitic in birds (Niewiadomska, 2002f). The taxonomic history of *Cardiocephaloides* is somewhat complex. The majority of the current members of *Cardiocephaloides* were originally placed in the genus *Cardiocephalus* Szidat, 1928. Sudarikov (1959) erected *Cardiocephaloides* for *Cardiocephaloides brandesii* (Szidat, 1928); while Dubois (1968) viewed *Cardiocephaloides* as a synonym of *Cardiocephalus*. However, Dubois (1970a) found the genus name *Cardiocephalus* to be pre-occupied and restored the name *Cardiocephaloides* for members of *Cardiocephalus*. At present, this genus of strigeids contains seven recognized species: the type-species *Cardiocephaloides longicollis* (Rudolphi, 1819), *C. brandesii*, *Cardiocephaloides hilli* (Johnston, 1904), *Cardiocephaloides medioconiger* (Dubois et Perez-Vigueras, 1949) (syn. *Cardiocephalus megaloconus* [Cable, Connor et Balling, 1960]), *Cardiocephaloides musculosus* (Johnston, 1904), *Cardiocephaloides ovicorpus* Dubois et Angel, 1972 and *Cardiocephaloides physalis* (Lutz, 1926) (syn. *Cardiocephalus szidati* Hartwich, 1954) (Dubois, 1968, 1982; Dubois & Angel, 1972).

While most *Cardiocephaloides* species are parasitic in larid birds as adults, *C. physalis* uniquely parasitizes penguins. *Cardiocephaloides physalis* was originally described from the Magellanic penguin *Spheniscus magellanicus* (Forster) collected near the coasts of Uruguay and Brazil (Lutz, 1926; Dubois, 1968). It was subsequently reported from *S. magellanicus* in Argentina, Chile and Peru (e.g., Díaz et al., 2010; Brandão et al., 2013; Fernández et al., 2019), the Humboldt penguin *Spheniscus humboldti* Meyen in Chile and Peru (González-Acuña et al., 2008; Angulo-Tisoc et al., 2018) and the African penguin *Spheniscus demersus* (Linnaeus) in South Africa (e.g., Randall & Bray, 1983; Horne et al., 2011). In addition, *C. physalis* was rarely reported from some non-penguin hosts in the Neotropics: the gray gull *Leucophaeus modestus* Tschudi, the Guanay cormorant *Phalacrocorax bougainvilleii* (Lesson) and the sooty shearwater *Puffinus griseus* (Gmelin) in Peru (e.g., Lutz, 1928; Tantalean et al., 1992). Despite being commonly reported in ecological surveys of helminths of penguins, it has never been included in a molecular phylogenetic analysis.

Herein, we provide novel DNA sequences of the entire ITS region (ITS1 + 5.8S + ITS2) and partial 28S rRNA gene of the nuclear ribosomal DNA as well as partial sequences of the mitochondrial cytochrome *c* oxidase 1 (*cox*1) gene from 3 species of *Cardiocephaloides* including *C. physalis* from *S. magellanicus* collected in Chile. We use newly generated and previously available DNA sequences to examine the phylogenetic interrelationships of 5 *Cardiocephaloides* spp.

Materials & Methods

Specimens

We obtained adult specimens of Cardiocephaloides from frozen carcasses of two *S. magellanicus* that died in the natural monument "Los Pingüinos" (55°55′08″S; 70°34′37″W) in Chile during winter of 2018, as well as from a royal tern Thalasseus maximus Boddaert from Mississippi, U.S.A. and European herring gulls Larus argentatus Pontoppidan from Kherson and Kyiv oblasts, Ukraine (Table 14). Dead digeneans from frozen and thawed carcasses of penguins were immediately fixed in 70% or 95% ethanol.

Phylogenetic analyses

Phylogenetic relationships of Cardiocephaloides spp. were analyzed using 28S, the ITS1 region (i.e., ITS1 + partial 5.8S sequences) and cox1 datasets as separate alignments. Newly obtained and previously published sequences were aligned using ClustalW implemented in MEGA7 (Kumar at al., 2016); alignments were trimmed to the length of the shortest sequence. Suchocyathocotyle crocodili (Yamaguti, 1954) was selected as outgroup in the 28S analysis based on the topology presented by Achatz et al.

Table 14. *Cardiocephaloides* spp. sequenced in this study including hosts, geographic origin, GenBank and museum accession numbers. HWML: Harold W. Manter Laboratory.

Digenean taxa	Host species	Geographic origin	Museum No.	Accession numbers		
				28S	ITS1 + 5.8S + ITS2	*cox*1
Cardiocephaloides longicollis	*Larus argentatus*	Ukraine	HWML-216111	MN820662	MN820662	MN817944
C. longicollis	*L. argentatus*	Ukraine	–	MN820663	MN820663	MN817945
C. medioconiger	*T. maximus*	U.S.A.	–	MN820664	MN820664	MN817946
Cardiocephaloides physalis	*Spheniscus magellanicus*	Chile	HWML-216112, HWML-111448	MN820665	MN820665	MN817947

(2019d). *Cotylurus marcogliesei* Locke, Van Dam, Caffara, Pinto, Lopez-Hernandez et Blanar, 2018 was selected as outgroup in the ITS1 region and *cox*1 analyses based on the results of our analysis of 28S and the availability of ITS region and *cox*1 sequences from the same isolate.

The 28S alignment included newly generated sequences of *C. longicollis*, *C. medioconiger* and *C. physalis* and previously published sequences of *C. medioconiger* and an unidentified *Cardiocephaloides* sp. along with 16 members of the Diplostomidae Poirier, 1886, two members of the Proterodiplostomidae Dubois, 1936 and 10 other members of the Strigeidae. The ITS1 region and *cox*1 alignments included newly generated sequences of *C. longicollis*, *C. medioconiger* and *C. physalis* along with previously published sequences of *C. medioconiger* and *Cardiocephaloides* sp. In addition, the ITS1 alignment included a sequence of a previously unidentified strigeid that proved to be a member of *Cardiocephaloides* based on a BLAST search and our preliminary phylogenetic analyses.

Independent phylogenetic analyses were conducted using Bayesian Inference (BI) as implemented in MrBayes Ver. 3.2.6 software (Ronquist & Huelsenbeck, 2003). The general time-reversible model with estimates of invariant sites and gamma-distributed among-site variation (GTR + I + G) was determined as the best-fitting nucleotide substitution model using MEGA7 software for each alignment (Kumar et al., 2016). The BI analyses were performed using MrBayes software as follows: Markov chain Monte Carlo (MCMC) chains were run for 3,000,000 generations with sample frequency set at 1,000. Log-likelihood scores were plotted and only the final 75% of trees were used to produce the consensus trees by setting the "burn-in" parameter at 750. This number of generations was considered sufficient because the SD dropped below 0.01. The trees were visualized in FigTree ver. 1.4 software (Rambaut, 2016) and

annotated in Adobe Illustrator®. Pairwise comparisons for each locus were carried out with assistance of MEGA7 (Kumar et al., 2016).

Results & Discussion

Molecular phylogenies

The 28S alignment was 1,135 bp long; 3 nucleotide positions were excluded due to indels. The phylogenetic tree resulting from the BI of the 28S alignment demonstrated the non-monophyletic nature of the two major diplostomoidean families the Diplostomidae and Strigeidae (Fig. 23) similar to the results of several previous studies (e.g., Achatz et al., 2019c, d; Blasco-Costa & Locke, 2017; Hernández-Mena et al., 2017; Queiroz et al., 2020). All members of *Cardiocephaloides* formed a very strongly supported clade (100%) with fully resolved internal topology. *Cardiocephaloides longicollis* formed a separate branch within this clade (Fig. 23) from a 99% supported clade of *C. physalis* + (*C. medioconiger* + *Cardiocephaloides* sp.). Both sequences of *C. medioconiger* formed a 100% supported clade.

The interrelationships within *Cardiocephaloides* were additionally studied using the 742 bp long ITS1 region alignment and 462 bp long *cox*1 alignment; 63 nucleotide positions were excluded due to ambiguous homology, mostly indels. The phylogenetic tree resulting from the BI analysis of ITS1 region had somewhat different topology than the *Cardiocephaloides* clade in the phylogeny based on 28S alone (Fig. 24). *Cardiocephaloides physalis* formed a sister group to the other *Cardiocephaloides* species

Cardiocephaloides sp. from New Zealand (GenBank accession KU695784) appeared as an unsupported clade nested among the remainder of the members of the genus.

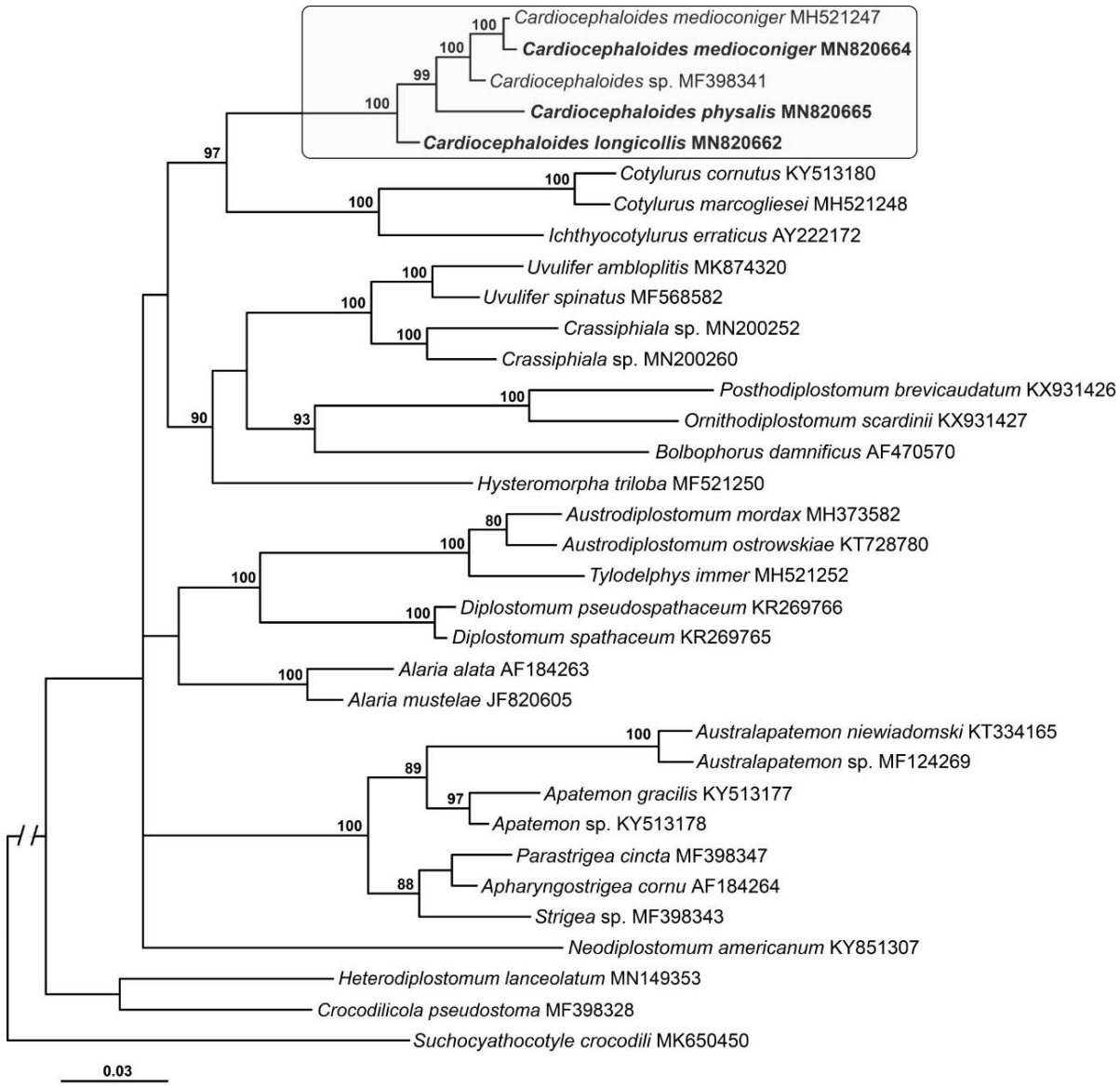

Figure. 23 Phylogenetic position and interrelationships of *Cardiocephaloides* spp. among diplostomoideans based on Bayesian Inference (BI) analysis of partial 28S rRNA gene sequences. Members of *Cardiocephaloides* are indicated by the shaded rectangle. Bayesian Inference posterior probability values lower than 80% are not shown. New sequences obtained in this study are in bold. Scale bar indicates number of substitutions per site. (after Achatz et al., 2020b)

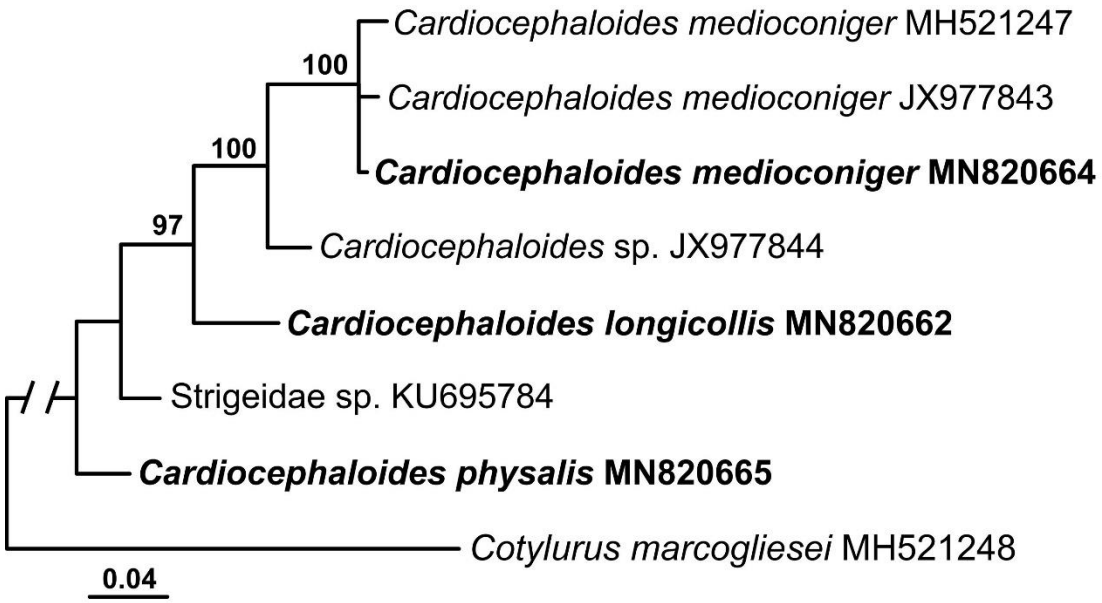

Figure. 24 Phylogenetic interrelationships among 5 species of *Cardiocephaloides* included in our study based on Bayesian Inference (BI) analysis of the partial ITS1 + 5.8S region sequences. Bayesian Inference posterior probability values lower than 80% are not shown. New sequences obtained in this study are in bold. Scale bar indicates number of substitutions per site. (after Achatz et al., 2020b)

Cardiocephaloides longicollis formed a 97% supported clade with a strongly supported clade (100%) of *Cardiocephaloides* sp. + *C. medioconiger*. The three sequences of *C. medioconiger* formed a 100% supported clade. Since ITS2 sequences of the unidentified *Cardiocephaloides* from New Zealand were not available, we performed a BI analysis (not shown) including the ITS1 + 5.8S + ITS2 regions. This analysis did not reveal any meaningful changes in topology or level of supports of *Cardiocephaloides* species.

The branch topology in the phylogenetic tree resulting from the BI analysis of *cox*1 sequences was somewhat different from both phylogenies based on sequences of ribosomal DNA (Fig. 25). *Cardiocephaloides physalis* formed the first branch followed by a strongly supported clade (100%) of *Cardiocephaloides* sp. (GenBank accession JX977784) + an unsupported clade of *C. longicollis* + *C. medioconiger*. The two unique sequences of *C. longicollis* and the five unique sequences of *C. medioconiger* both formed separate 100% supported clades.

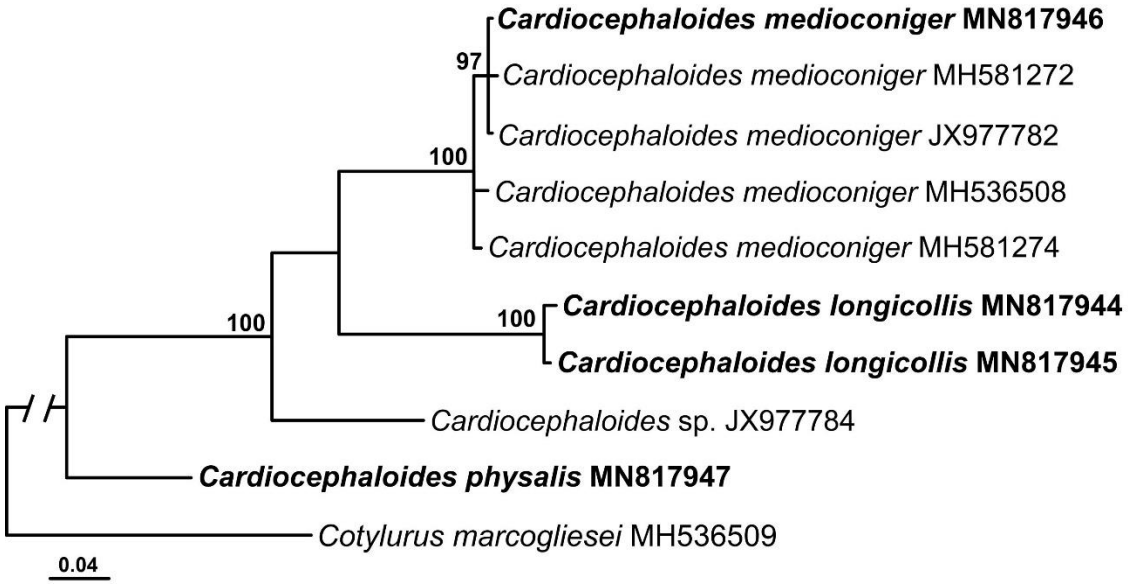

Figure. 25 Phylogenetic interrelationships among 4 species of *Cardiocephaloides* included in our study based on Bayesian Inference (BI) analysis of partial *cox*1 mtDNA gene sequences. Bayesian Inference posterior probability values lower than 80% are not shown. New sequences obtained in this study are in bold. Scale bar indicates number of substitutions per site. (after Achatz et al., 2020b)

Genetic variation

Pairwise nucleotide comparisons of partial 28S sequences among *Cardiocephaloides* spp. are provided in Table 15. The interspecific divergence in 28S sequences of *Cardiocephaloides* spp. was generally low at 0.4–1.6% (5–18 bp out of 1,151). The previously published sequences of *C. medioconiger* (GenBank accession MH521247) and *Cardiocephaloides* sp. (GenBank accession MF398341) were the least divergent (0.4%). In contrast, our new sequences of *C. medioconiger* (GenBank accession MN820664) and *C. physalis* (GenBank accession MN820665) demonstrated the greatest divergence (1.6%). The interspecific divergence level among sequences of 28S for *Cardiocephaloides* spp. included in this study was similar to that demonstrated within other genera of diplostomoideans, e.g., *Australapatemon* Sudarikov, 1959 (0–1.2%), *Braunina* Wolf, 1903 (0.16%), *Crassiphiala* Van Haitsma, 1925 (0.2–2.4%), *Parastrigea* Szidat, 1928 (0.1–0.7%) and *Uvulifer* Yamaguti, 1934 (0.1–2.2%) (Gordy et

Table 15. Pairwise comparisons of partial sequences of the 28S rRNA gene among *Cardiocephaloides* species included in this study. Percentage differences are given above diagonal and the number of variable nucleotide positions are given below the diagonal. The alignment was 1,151 bp long. Isolate identifiers are given in parentheses after species names.

	1. MN820662	**2.** MH521247	**3.** MN820664	**4.** MN820665	**5.** MF398341
1. *Cardiocephaloides longicollis* (VT463) MN820662	–	1.2%	1.3%	1.5%	1%
2. *Cardiocephaloides medioconiger* (C.IN.FKY.Tm.3115.1) MH521247	14	–	0.1%	1.5%	0.4%
3. *C. medioconiger* (EP521) MN820664	15	1	–	1.6%	0.5%
4. *Cardiocephaloides physalis* (VT6424) MN820665	17	17	18	–	1.4%
5. *Cardiocephaloides* sp. (DNA181) MF398341	11	5	6	16	–

Table 16. Pairwise comparisons for the 1,080 bp long alignment of the ribosomal ITS1 + 5.8S + ITS2 region and the 628 bp long alignment of the ITS1 among *Cardiocephaloides* spp. included in this study. Percentage differences are given above diagonal and the number of variable nucleotide positions are given below the diagonal. Values for the ITS1 + 5.8S + ITS2 region are given before "/" and values for only the ITS1 are given after "/". Isolate identifiers are given in parentheses after species names.

	1. MN820662	**2.** MH521247	**3.** JX977843	**4.** MN820664	**5.** MN820665	**6.** JX977844	**7.** KU695784
1. *Cardiocephaloides longicollis* (VT463) MN820662	–	2.8%/3.3%	2.7%/3.2%	2.7%/3.2%	5.9%/8.8%	2.8%/2.7%	NA/2.9%
2. *Cardiocephaloides medioconiger* (C.IN.FKY.Tm.3115.1) MH521247	30/21	–	0.2%/0.3%	0.2%/0.2%	6.9%/9.9%	1.9%/1.9%	NA/3.8%
3. *C. medioconiger* (DNA594) JX977843	29/20	2/2		0.2%/0.2%	6.9%/9.7%	1.9%/1.9%	NA/3.7%
4. *C. medioconiger* (EP521) MN820664	29/20	2/1	2/1	–	6.9%/9.7%	1.9%/1.8%	NA/3.7%
5. *Cardiocephaloides physalis* (VT6424) MN820665	64/55	75/62	74/61	75/61	–	6.9%/9.4%	NA/6.8%
6. *Cardiocephaloides* sp. (DNA181) JX977844	30/17	20/12	20/12	20/11	75/59	–	NA/3.5%
7. Strigeidae sp. (MB.CA.5) KU695784	NA/18	NA/24	NA/23	NA/23	NA/43	NA/2	–

al., 2017; Hernández-Mena et al., 2017; Achatz et al., 2019a, c, d). Our new 28S sequence of *C. medioconiger* had a single base difference (0.1%) from the previously published sequence (GenBank accession MH521247) available in GenBank. No differences were detected between the 28S sequences of our two *C. longicollis* isolates from *L. argentatus*.

Pairwise nucleotide comparisons of the ITS region and ITS1 sequences among *Cardiocephaloides* spp. are provided in Table 16. Sequences of ITS1 + 5.8S + ITS2 and ITS1 were compared separately to include the unidentified *Cardiocephaloides* sp. from New Zealand which did not have ITS2 sequences. The interspecific divergence of sequences for the ITS region was 1.9–6.9% (20–75 bp out of 1,080) among sequenced *Cardiocephaloides* species. *Cardiocephaloides* sp. (GenBank accession JX977844) and the *C. medioconiger* sequences (GenBank accessions JX977843, MH521247, MN820664) were the most similar (1.9%), while *C. physalis* (GenBank accession MN820665) and *C. medioconiger* sequences (GenBank accessions JX977843, MH521247, MN820664) demonstrated the highest levels of divergence (6.9%). The greater interspecific differences between *C. physalis* and other *Cardiocephaloides* spp. was largely due to 33 bp long insertion in ITS1 which often represented almost half of the differences. The level of interspecific differences observed among the ITS region sequences of *Cardiocephaloides* spp. included in this study was similar to that reported for some diplostomoidean genera, e.g., *Diplostomum* von Nordmann, 1832 (1.8–4.6%), *Uvulifer* (2–7.8%), *Posthodiplostomum* Dubois, 1936 (6–9.3%) and *Tylodelphys* Diesing, 1850 (0.7–8.3%) (Galazzo et al., 2002; Blasco-Costa & Locke, 2017; López-Hernández et al., 2018; López-Jiménez et al., 2018). At the same time, the level of interspecific differences of the ITS region sequences among *Cardiocephaloides* spp. was somewhat higher than in some other some other diplostomoidean genera, e.g., *Australapatemon* (0.4–1.9%), *Hysteromorpha* Lutz, 1931 (0.1–

1.3%), *Ornithodiplostomum* Dubois, 1936 (0–1.3%) (Gordy et al., 2017; Locke et al., 2018; López-Hernández et al., 2018). Interspecific differences among *Cardiocephaloides* spp. sequences of the ITS region sequences in our study is also somewhat similar to what was demonstrated by Locke et al. (2018) using only *C. medioconiger* (GenBank accession MH521247) and *Cardiocephaloides* sp. (GenBank accession JX977844) (2.3%). No differences were detected between the ITS1 + 5.8S + ITS2 sequences of our two *C. longicollis* isolates. Up to 0.2% difference was detected among 3 unique sequences of the ITS region of *C. medioconiger.*

The interspecific divergence of ITS1 sequences among *Cardiocephaloides* spp. was 1.8–9.9% (11–62 bp out of 628), similar to that observed in the ITS region (1.9–6.9%). *Cardiocephaloides* sp. (GenBank accession JX977844) and *C. medioconiger* (GenBank accession MN820664) were the most similar (1.8%), while *C. physalis* (GenBank accession MN820665) and *C. medioconiger* (GenBank accession MH521247) demonstrated the greatest divergence level (9.9%).

Our new ITS2 sequences of *C. longicollis* from *L. argentatus* collected in Ukraine (GenBank accessions MN820662, MN820663) were identical to the two previously published (Born-Torrijos et al., 2016) sequences of *C. longicollis* from *Larus michahellis* Naumann (GenBank accessions KT454990, KT454991) in Spain. However, our new sequences of had up to 4% difference compared to the partial 5.8S sequences of the Spanish isolates along with up to 2% difference in partial 28S sequences. The few differences in the flanking regions of 5.8S and 28S between our sequences of *C. longicollis* and those from Spain can be likely explain by errors in the sequences from Spain because these region are completely identical in all other available sequences of *Cardiocephaloides*.

Pairwise nucleotide comparisons of partial *cox*1 sequences among *Cardiocephaloides* spp. are provided in Table 17. The *cox*1 sequences had a much greater interspecific divergence than the ribosomal sequences of the same species (8.7–11.8%; 34–46 bp out of 389). Previously published sequences of *C. medioconiger* (GenBank accession MH581274) + *Cardiocephaloides* sp. (GenBank accession JX977784) and *C. medioconiger* (GenBank accession MH536508) + *C. longicollis* (GenBank accession MN817945) were most similar in this gene (8.7%), while *C. longicollis* (GenBank accession MN817944) + *C. physalis* (GenBank accession MN817947) and *C. medioconiger* (GenBank accession MH581272) + *C. physalis* (GenBank accession MN817947) had the greatest divergence level (11.8%). Interspecific differences among sequences of *cox*1 for *Cardiocephaloides* spp. included in this study (8.7–11.8%) were somewhat similar to those reported for a number of other diplostomoidean genera, e.g., *Alaria* Schrank, 1788 (8.4–13.1%), *Australapatemon* (6.7–14.4%), *Braunina* (16.4%), *Cotylurus* Szidat, 1928 (3.4–10.8%), *Crassiphiala* (11–19.8%), *Hysteromorpha* (6.9–9.7%), *Parastrigea* (9.31–11.47%), *Posthodiplostomum* (15–22%), *Uvulifer* (9.3–15.3%), *Tylodelphys* (8–16.5%) (Hernández-Mena et al., 2014; Blasco-Costa et al., 2017; Gordy et al., 2017; Locke et al., 2018; López-Hernández et al., 2018; Achatz et al., 2019a, c, d). The interspecific differences among sequences of *cox*1 for *Cardiocephaloides* spp. were slightly greater than those demonstrated by Locke et al. (2018) using only *C. medioconiger* and *Cardiocephaloides* sp. (isolate DNA181) (7.4–9.7%). The intraspecific variability among *cox*1 sequences of different isolates of *C. medioconiger* was up to 1.3% (5 bp out of 389) and 0.3% (1 bp out of 389) between isolates of *C. longicollis*.

Table 17. Pairwise comparisons of partial sequences of the *cox*1 mtDNA gene among *Cardiocephaloides* species included in this study. Percentage differences are given above diagonal and the number of variable nucleotide positions are given below the diagonal. Results are based on a 389 bp long alignment. Isolate identifiers are given in parentheses after species names.

	1. MN817944	**2.** MN817945	**3.** MH536508	**4.** MH581272	**5.** MH581273	**6.** MH581274	**7.** JX977782	**8.** MN817946	**9.** MN817947	**10.** JX977784
1. *Cardiocephaloides longicollis* (VT463) MN817944	–	0.3%	9%	10%	9.5%	9.3%	9.8%	9.8%	11.8%	9.8%
2. *Cardiocephaloides longicollis* (VT7189) MN817945	1	–	8.7%	9.8%	9.3%	9%	9.5%	9.5%	11.6%	9.8%
3. *Cardiocephaloides medioconiger* (C.IN.FKY.Tm.3115.1) MH536508	35	34	–	1.3%	1%	0.8%	1%	1%	11.1%	9.5%
4. *C. medioconiger* (Cm.IN.FKY.Tm.3116 .3) MH581272	39	38	5	–	1.3%	1%	0.3%	0.3%	11.8%	9%
5. *C. medioconiger* (Cm.IN.FKY.Tm.3116 .2) MH581273	37	36	4	5	–	0.3%	1%	1%	11.1%	9%
6. *C. medioconiger* (Cm.IN.FKY.Tm.3116 .1) MH581274	36	35	3	4	1	–	0.8%	0.8%	10.8%	8.7%
7. *C. medioconiger* (DNA593) JX977782	38	37	4	1	4	3	–	0%	11.6%	9.3%
8. *C. medioconiger* (EP521) MN817946	38	37	4	1	4	3	0	–	11.6%	9.3%
9. *Cardiocephaloides physalis* (VT6424) MN817947	46	45	43	46	43	42	45	45	–	9.8%
10. *Cardiocephaloides* sp. (DNA181) JX977784	38	38	37	35	35	34	36	36	38	–

General remarks

This is the first study to generate DNA sequence data from *C. physalis* from South America and to include *C. physalis* in a molecular phylogeny. A shorter (954 base pairs long) 28S sequence of *C. physalis* from a fish intermediate host *Sardinops sagax* (Jenyns) collected in South Africa which is available in GenBank is identical to our sequence of *C. physalis* from *S. magellanicus* collected in Chile. While this may suggest that the two forms sequenced from African and South American coasts represent the same broadly distributed species, a comparison of faster mutating genes (e.g., *cox*1) would be more conclusive.

It is worth noting that *C. physalis* was nested within the *Cardiocephaloides* clade in the 28S tree suggesting potential secondary host switching from larids to penguins in the evolution of *Cardiocephaloides*. Molecular phylogenetic studies have demonstrated a relatively recent divergence and radiation of penguins (e.g., Subramanian et al., 2013; Prum et al., 2015). The very low diversity of diplostomoideans in penguins compared to other fish-eating birds (e.g., Fernandes et al., 2015) reflects both the loss of ancestral parasite fauna and insufficient evolutionary time to evolve a more diverse novel diplostomoidean fauna specific to penguins. Parasitism of *C. physalis* in avian hosts other than penguins has been reported very rarely, typically within the distribution of penguins (e.g., Lutz, 1928; Tantalean et al., 1992; Fernandes et al., 2015). However, the ability of *C. physalis* to infect a variety of hosts along with its morphological similarity with its relatives from other birds suggest the transition to parasitism in penguins likely happened relatively recently in the evolutionary history of the genus.

In our analysis of partial 28S sequences *C. longicollis* from the Palearctic appeared as a sister group to the clade that included all the remaining *Cardiocephaloides* spp. collected in the Nearctic and Neotropics (Fig. 23; Table 14). In the ITS1 region tree (Fig. 24) the unidentified

species from New Zealand appears as the second most basal taxon in the *Cardiocephaloides* tree next to *C. physalis*, albeit with low support. Based on its phylogenetic position, the adult stage of the New Zealand form may be parasitic in either penguins inhabiting the area, or other fish-eating birds. Therefore, it is intriguing to find out how the addition of DNA sequences of *Cardiocephaloides* spp. (preferably adults) from Australia might impact the tree topology of *Cardiocephaloides*. The Australian species need to be included in the future molecular phylogenetic studies to further explore the evolution and historical biogeography of this diplostomoidean lineage.

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Associated publication

Achatz, T. J., Pulis, E. E., González-Acuña, D. and Tkach, V. V. 2020b. Phylogenetic relationships of *Cardiocephaloides* spp. (Digenea, Diplostomoidea) and the genetic characterization of *Cardiocephaloides physalis* (Lutz, 1926) from Magellanic Penguin, *Spheniscus magellanicus* (Forster), in Chile. Acta Parasitologica 65: 525–534.

CHAPTER X

CONCLUSIONS

Molecular phylogenetic analyses of partial 28S sequences from diplostomoidean taxa clearly demonstrate the non-monophyly of the Cyathocotylidae, Diplostomidae and Strigeidae. While the present studies have begun to propose revisions to the system of the superfamily, additional thorough morphological studies of adult and larval diplostomoideans are required to properly re-evaluate the status of the Diplostomidae and Strigeidae. The molecular phylogenetic analyses (primarily based on 28S) demonstrated numerous host-switching events during the evolutionary history of the Diplostomoidea along with evidence of multiple dispersal events between biogeographic realms. Our results allowed establishment of 1 new subfamily and 3 new genera and revealed at least 8 new species of diplostomoideans.

Conclusion 1

The phylogenetic and morphological analyses of the Cyathocotylidae and Brauninidae demonstrated that members of *Braunina* belong to the Cyathocotylidae, thus placing the Brauninidae into synonymy of the Cyathocotylidae. In addition, our DNA sequences supported the presence of a second species in the currently monotypic *Braunina*. Our phylogeny revealed that *Cyathocotyle* spp. from crocodilians belong to a separate genus (*Suchocyathocotyle*, previously a subgenus) and subfamily (Suchocyathocotylinae subfam. n.). Our results revealed at

least two major host switching events and more than one transition between freshwater and marine environments in the evolutionary history of the Cyathocotylidae.

Conclusion 2

Phylogenetic analyses of novel 28S and *cox*1 sequence data of from a broad range of proterodiplostomid taxa has challenged the current systematic framework. As the result of our study: (i) the current subfamily based structure of the Proterodiplostomidae was abolished; (ii) three new genera, *Paraproterodiplostomum* n. g., *Neocrocodilicola* n. g. and *Proteroduboisia* n. g. were described and *Pseudoneodiplostomoides* Yamaguti, 1954 was restored and elevated from sub-genus to genus level; (iii) two new species, *Paraproterodiplostomum currani* n. g., n. sp. and *Archaeodiplostomum overstreeti* n. sp., were described from American alligator in Mississippi, U.S.A. Our analysis did not support the use of the structure of terminal ducts of the reproductive system for differentiation among sub-families within the family, although they proved to be useful for distinguishing among genera and species. Our molecular phylogeny of the Proterodiplostomidae closely matched the current molecular phylogeny of crocodilians. A key to proterodiplostomid genera was provided.

Conclusion 3

We described 2 new species of *Uvulifer* from Peru (*Uvulifer batesi* n. sp. and *Uvulifer pequenae* n. sp.) based on the combination of morphological and molecular data. In addition, we used newly generated sequence data to differentiate among species and examine phylogenetic affinities of *Uvulifer*. Our 28S analysis revealed at least 6 well-supported clades within the genus. Our 28S phylogeny did not reveal any clear patterns of host association between *Uvulifer*

and particular species of kingfishers; however, it indicated at least 2 independent geographical dispersal events in the evolutionary history of the New World *Uvulifer*. Our results clearly demonstrated that the diversity of *Uvulifer* in the New World has been underestimated.

Conclusion 4

Our molecular and morphological study of adult and larval crassiphialines from the Americas revealed the presence of at least 3 species-level lineages of *Crassiphiala* from the Nearctic and 2 species-level lineages from the Neotropics. This is the first record of *Crassiphiala* from the Neotropics. The results of our analyses do not support the monophyly of the Crassiphialinae. In addition, our results clearly demonstrate that the diversity of *Crassiphiala* has been underestimated.

Conclusion 5

The presence of a progenetic metacercaria in *Codonocephalus* along with its morphology atypical for diplostomids were reflected in its phylogenetic position as an independent branch among other major lineages of the highly diverse Diplostomoidea.

Conclusion 6

Our molecular and morphological study of *Sphincterodiplostomum* specimens from birds and caimans revealed the presence of at least 2 species of *Sphincterodiplostomum* in the Neotropics, one of which we described as a new species. We provided the first molecular phylogeny of the Diplostomoidea that includes *Sphincterodiplostomum*. Our study provided the

first record of *Sphincterodiplostomum musculosum* from caimans, along with the first record of fully mature adult *S. musculosum* from green kingfisher *Chloroceryle americana*.

Conclusion 7

Cardiocephaloides as represented in the currently available dataset is monophyletic. Parasitism in penguins of *Cardiocephaloides physalis* is likely the result from a secondary host-switching event. Identical 28S sequences of *Cardiocephaloides physalis* from South America and Africa cautiously confirmed the broad distribution of this species, although comparison of faster mutating genes (e. g., *cox*1) is recommended for a better substantiated conclusion.

Additional research conducted

Besides the research on the systematics and taxonomy of diplostomoideans, I worked on a diversity of other research topics during my doctoral studies. I have been a co-author on 4 peer-reviewed publications (in review or published) on members of the digenean family Dicrocoeliidae (Achatz et al., 2018, 2020a; Tkach et al., 2018; Fernandes et al., *in review*) along with 3 peer-reviewed publications on ecology and population genetics of digeneans (McAllister et al., 2019; Calhoun et al., 2020; Johnson et al., 2020). In addition, I have been involved in research on avian malaria, Lyme disease in tick and small mammal populations, and Zika virus

List of peer-reviewed publications

Achatz, T. J., Bell, J. A., Melo, F. T. V., Fecchio, A. and Tkach, V. V. 2021. Phylogenetic position of *Sphincterodiplostomum* Dubois, 1936 (Digenea: Diplostomoidea) with description of a second species from Pantanal, Brazil. Journal of Helminthology 95: e6.

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